Effects of forest expansion on mountain grassland: changes within soil organic carbon fractions

Claudia Guidi · Jakob Magid · Mirco Rodeghiero · Damiano Gianelle · Lars Vesterdal

Abstract

Background and aims Grassland abandonment followed by forest succession is the dominant land-use change in the European Alps. We studied the impact of current forest expansion on mountain grassland on changes in physical soil organic carbon (SOC) fractions along a land-use and management gradient, focusing on changes in aggregate stability and particulate organic matter (POM).

Methods Four successional stages were investigated: managed grassland, two transitional phases in which grassland abandonment led to colonization by *Picea abies* (L.) Karst., and old mixed forest dominated by *Fagus sylvatica* L. and *P. abies*. Soil samples collected from the mineral soil (0–5 cm, 5–10 cm, 10–20 cm) were fractionated following two procedures: 1) aggregate size fractionation, separating aggregates based on their dimension, and 2) size-density fractionation, separating stable aggregates from non-occluded POM.

Results The dimension of aggregates assessed by aggregate size fractionation tended to increase, whereas SOC allocation to stable aggregates assessed by size-density fractionation decreased following conversion of grassland to forest (e.g. from 81 to 59 % in the 0–5 cm layer). The amount of SOC stored in POM increased by 3.8 Mg ha^{-1} in the integrated 0–20 cm layer from managed grassland to old forest.

Conclusions The combination of two physical SOC fractionation procedures revealed that natural forest succession on abandoned grasslands led to a decline in physical SOC stability in the mineral soil, suggesting that SOC can become more susceptible to management and environmental change.

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Introduction

The abandonment of grasslands represents the dominant land-use change (LUC) and the main driver for the establishment of new forest areas in the European Alps (Zimmermann et al. 2010). While fertile grasslands

were more intensively managed, the less productive and accessible grasslands have been progressively abandoned (Tasser and Tappeiner 2002), followed by tree establishment as a result of natural succession (Haugo et al. 2013). In alpine regions, forest expansion on abandoned grasslands was reported to decrease soil organic carbon (SOC) stored in mineral layers by Thuille et al. (2000), Thuille and Schulze (2006) and Alberti et al. (2008), but no change or an increase in SOC stocks were also shown (Montane et al. 2007; Risch et al. 2008). In Trentino, a region located in the Southern Alps (Italy), a recent study investigating natural succession of forest on abandoned grasslands revealed that mineral SOC stocks decreased, although total SOC stocks did not change significantly (Guidi et al. 2014).

The response of soil organic matter (SOM) stability, which allows protection against mineralization (Kögel-Knabner et al. 2008), is largely unknown for forest expansion on abandoned grasslands in mountainous regions, as changes in SOM stability may follow different directions from trends in bulk SOM (Creamer et al. 2011). The mechanisms of SOM stabilization include organic matter (OM) adsorption to clay and silt particles, occlusion within aggregates, and biochemical recalcitrance (Dungait et al. 2012). Since the importance of biochemical recalcitrance as a mechanism of C stabilization in soil has been questioned (Dungait et al. 2012), the spatial arrangement of OM within the soil matrix can be identified as one of the main drivers of SOM dynamics, determining physical accessibility of SOM to decomposers and extracellular enzymes (Kögel-Knabner et al. 2008; Schmidt et al. 2011).

Physical fractionation procedures can be applied to unravel responses of SOM stability to land-use and management changes, through separation of SOM into discrete portions (Cambardella and Elliott 1992; Six et al. 1998; von Lützow et al. 2007). Methods to fractionate SOM can combine several size and density separation steps (Magid et al. 1996; Six et al. 2002; John et al. 2005; Zimmermann et al. 2007). Different intensities of soil dispersion can separate aggregates characterized by different physical stabilities, thus the application of more fractionation procedures to the same soil can offer a more comprehensive view of changes in physical SOM stability (Moni et al. 2012).

It remains unclear how physical SOM fractions respond to forest expansion on abandoned grasslands as previous studies often focused on human-induced

afforestation (Six et al. 2002; Poeplau and Don 2013) or on the first phases of grassland abandonment before considerable forest expansion (Meyer et al. 2012).

A shift from grassland to forest can affect physical OM protection in soil aggregates through changes in the quantity and quality of the plant input (Guo et al. 2007; Hiltbrunner et al. 2013), in the soil decomposer community (Macdonald et al. 2009) and in the microclimatic conditions (Nosetto et al. 2005). On the one hand, the formation of large macroaggregates can be favored by roots with larger diameter (Jastrow et al. 1998), i.e. roots of trees compared to those of grasses (Jackson et al. 1997). Moreover, as fungal hyphae can physically tie together macroaggregates (Oades 1984), a shift from a bacterial- to a fungal-dominated microbial community following grassland abandonment and forest development (Zeller et al. 2000; Macdonald et al. 2009) can contribute to increase the formation of large macroaggregates in forest sites. This can lead to an increase in the mean weight diameter (MWD) of aggregates, which provides a synthetic measure of aggregate dimension in soil (Kemper and Rosenau 1986). On the other hand, the lower mass and turnover of fine roots in forests compared with grasslands (Guo et al. 2007; Solly et al. 2013) can lead to lower aggregate stability, given the positive effect of fine roots on aggregate stabilization through physical binding of aggregates or through the enhancement of microbial activity (Jastrow et al. 1998). Moreover, reduced quality (e.g. nutrient and lignin contents) of both aboveground and belowground input (Hiltbrunner et al. 2013) together with a decrease in soil temperature caused by forest expansion (Nosetto et al. 2005) hamper OM decomposition processes, leading to a further decrease in the production of aggregate-stabilizing agents (Cotrufo et al. 2013). Due to a slowdown of OM decomposition processes following the natural succession from grassland to forest, OM inputs are subject to reduced microbial processing, leading to decreased OM incorporation into soil aggregates and thus to accumulation of particulate organic matter (POM) not occluded within aggregates (Gregorich et al. 2006). Changes in physical SOM fractions following LUC are generally more evident in the upper soil layers, as deeper soil layers are affected to a lesser extent by changes in litter input (Poeplau and Don 2013).

The main objective of this study was to identify changes in physical SOC fractions following forest expansion on abandoned grassland in a mountainous region of the Alps. Based on the differences between

grassland and forest ecosystems such as root morphology and litter quality, we expect that the formation of larger aggregates and accumulation of POM would be favored in forest compared to grassland successional stages, whereas aggregate stability would decrease. Our specific objectives were therefore to evaluate: (i) changes in aggregate stability, using both conventional wet-sieving and a more intensive physical separation of aggregates, and (ii) changes in POM. These objectives were addressed by using two fractionation procedures to gain complementary information on physical SOM stability: aggregate size fractionation, to separate water-stable aggregates based on their dimension, and size-density fractionation, to separate aggregates that are stable toward physical disturbance from non-occluded POM. We investigated a land-use and management gradient located in Trentino (Southern Alps, Italy) comprising a managed grassland, mown and manured annually, two transitional phases in which grassland abandonment led to progressive colonization by Norway spruce (*Picea abies* (L.) Karst.), and an old mixed forest, considered as a reference for long-term forest land use and dominated by European beech (*Fagus sylvatica* L.) and Norway spruce (Guidi et al. 2014).

Methods

Study area

The study area was selected among the areas identified by the Neoboschi project (Sitzia 2009) in order to investigate a forest expansion pattern typical for Trentino region, located in the Southern Alps, Italy (Guidi et al. 2014). The study area has an elevation of around 1,150 m a.s.l. In the period 1990–2011, a mean annual temperature of 7.2 °C and a mean annual precipitation of 1,278 mm were registered, as recorded by the closest meteorological station. The rainiest months were October and November, while the driest month was February. The soil type is a Cambisol (IUSS 2007), developed on calcareous parent material and with a clay-loam to clay soil texture (Table 1). Four successional stages were compared: (i) managed grassland (manG), mown and manured for at least the past 100 years; (ii) abandoned grassland (abanG), where a successional community of shrubs and Norway spruce has developed since grassland was abandoned approximately 10 years ago; (iii) early-stage forest (earlyF),

dominated by Norway spruce, established in grasslands abandoned around 1970; and (iv) old mixed forest (oldF), composed mainly of European beech and Norway spruce and already present in the historical land register (1861). The successional stages manG-abanG-earlyF can be interpreted as phases of a LUC chronosequence, using a space for time substitution approach, with oldF as a reference. Further information about the study area can be found in Guidi et al. (2014).

Soil sampling and processing

Within a 4 ha area, three plots with similar climatic conditions, soil type and parent material were investigated for each successional stage (Guidi et al. 2014). In the study design, we tried to avoid pseudoreplication as much as possible, through interspersion of plots of the same successional stage with plots of a different successional stage, however plots of abanG tended to be clustered. Even though results will be interpreted as specific for the study area, the study area was selected in order to be representative as far as possible for forest expansion in Trentino region.

In each plot, eight soil cores (4.8 cm diameter) were sampled equidistantly on the perimeter of a circle with a 5 m-radius. Mineral soil samples were collected from 0–5 cm, 5–10 cm and 10–20 cm layers and were fractionated separately for the three layers to detect if the effect of LUC on physical fractions was more pronounced at specific soil depths, considering that LUC can affect litter and root distribution throughout the soil profile. Results obtained for the three layers were subsequently integrated over the 0–20 cm layer, to convey an overall picture of SOC distribution within physical fraction for the 0–20 cm layer. The organic layers were sampled and analyzed for C content by Guidi et al. (2014) and used for calculating total SOC stocks.

The field-moist soil samples were manually sieved at 8 mm, sorting out stones and roots. The samples collected at the same depth of each plot were pooled to obtain a composite sample and air-dried. The air-dried composite samples were split in two subsamples: the first for aggregate size fractionation and the second for size-density fractionation. We used two fractionation procedures in order to gain a more complete understanding of aggregate stability (Fig. 1). Aggregate size fractionation was applied to separate aggregates stable toward wet-sieving, while in size-density fractionation a stronger physical disturbance was applied, allowing to

Table 1 Mineral soil properties at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest)

	Clay (%)	Silt (%)	Sand (%)	pH	Inorganic C (mg g^{-1})	Organic C (mg g^{-1})	C/N	Root biomass (Mg ha^{-1})
manG								
0–5 cm	40 (3)	40 (1)	21 (2)	5.7 (0.6)	7 (5)	83 (3)	10.5 (0.1)	3.3 (1.0)
5–10 cm	39 (2)	38 (1)	23 (1)	5.6 (0.6)	8 (6)	50 (1)	10.0 (0.1)	1.1 (0.2)
10–20 cm	38 (3)	37 (1)	25 (1)	5.9 (0.6)	9 (8)	27 (2)	10.1 (0.1)	0.3 (0.1)
abanG								
0–5 cm	46 (1)	35 (2)	18 (2)	6.0 (0.1)	10 (3)	92 (4)	12.3 (0.3)	6.2 (0.7)
5–10 cm	46 (1)	32 (3)	22 (3)	6.4 (0.2)	4 (1)	58 (2)	11.0 (0.2)	1.7 (0.5)
10–20 cm	41 (3)	33 (1)	26 (2)	6.8 (0.1)	9 (1)	37 (1)	10.5 (0.2)	1.1 (0.2)
earlyF								
0–5 cm	48 (2)	34 (2)	18 (4)	5.8 (0.3)	8 (2)	70 (4)	13.0 (0.1)	6.8 (0.6)
5–10 cm	44 (1)	34 (1)	23 (1)	5.9 (0.5)	5 (2)	49 (4)	11.9 (0.4)	6.6 (0.7)
10–20 cm	41 (3)	35 (1)	24 (3)	6.3 (0.5)	10 (8)	30 (2)	11.0 (0.4)	3.3 (1.3)
oldF								
0–5 cm	42 (6)	33 (5)	24 (1)	4.7 (0.5)	11 (11)	57 (10)	15.1 (0.5)	6.8 (0.5)
5–10 cm	40 (3)	33 (2)	27 (2)	5.3 (0.7)	11 (11)	44 (9)	14.0 (0.6)	5.5 (0.5)
10–20 cm	41 (4)	32 (2)	27 (3)	5.9 (0.5)	15 (15)	34 (8)	13.1 (0.9)	7.2 (1.8)

Values are means (n=3) with standard error of the mean in brackets. Modified from Guidi et al. (2014)

obtain aggregates stable toward more pronounced physical action and to separate non-occluded POM. The subsample for size-density fractionation was further sieved at 2 mm, sorting out the coarse fraction (>2 mm) and hand-picking roots with length approximately greater than 2 cm. Roots separated during sieving at 2 mm were used together with roots collected during 8 mm-sieving for the determination of root biomass by oven drying at 105 °C to constant weight. Moreover, around 10 g of each composite sample were dried at 105 °C to constant weight for the determination of soil dry mass.
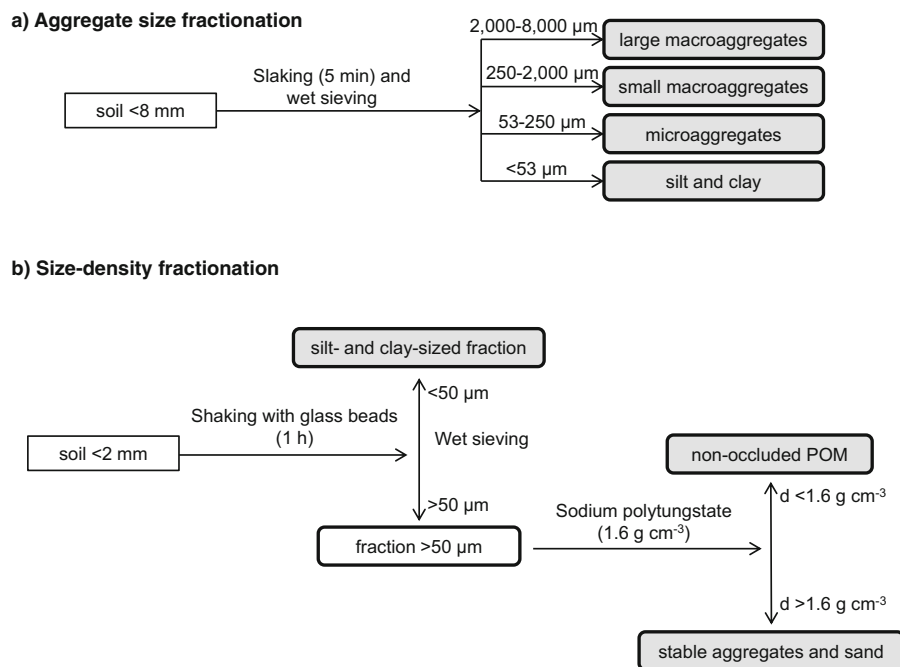
Aggregate size fractionation

A 80 g-subsample of soil <8 mm was wet-sieved through three sieves (2,000, 250, 53 μm mesh size) according to Cambardella and Elliott (1993), as shown in Fig. 1. Four aggregate size fractions were obtained: (i) large macroggregates (2,000–8,000 μm); (ii) small macroaggregates (250–2,000 μm); (iii), microaggregates (53–250 μm); and (iv) silt and clay fraction (<53 μm). The soil samples were submerged for 5 min in deionized water on the 2,000 μm-sieve, to allow slaking of

aggregates (Elliott 1986). After slaking, aggregate separation was accomplished manually, through immersion of the 2,000 μm-sieve in water 50 times during 2 min (Cambardella and Elliott 1993). The litter >2,000 μm that floated on water was separated from large macroaggregates according to Six et al. (1998), as not belonging to soil <2,000 μm. The material that passed through the 2,000 μm-sieve was transferred on the next sieve, and the process repeated, but litter that floated on water was retained in the corresponding aggregate size fraction. The fractions were dried in a forced-air oven at 55 °C for 24 h and weighed. Rock fragments were removed manually from large macroaggregates, as these fragments did not belong to soil <2,000 μm. The mass recovery was on average 99.9 % (SE=0.2 %, range 94.4–101.5 %).

Size-density fractionation

A size-density fractionation procedure, modified from Magid et al. (2002) and Magid et al. (2010) was applied to soil <2 mm, as shown in Fig. 1. The major modifications of the original procedure were: (i) soil dispersion was performed with glass beads instead of sodium

a) Aggregate size fractionation

b) Size-density fractionation

Fig. 1 Scheme of the aggregate size fractionation (**a**) and the size-density fractionation (**b**) procedure

hexametaphospate, which can lead to the solubilization of C and to its redistribution among fractions as reported by Cerli et al. (2012); (ii) shaking time was increased to 1 h as preliminary trials showed that 30 min were not sufficient to bring all soil material into suspension; (iii) soil was separated using only a 50 μm-sieve instead of 1,000, 100 and 50 μm-sieves, in order to separate only larger and stable aggregates from silt- and clay-sized particles; (iv) separation by density was performed only for the fraction >50 μm for operational simplicity.

A 25 g-soil subsample was shaken in 90 ml of deionized water with 12 glass beads (5 mm diameter) for 1 h in an end-over-end shaker at 30 rotations per minute. The soil suspension was then transferred to a 50 μm-sieve and washed with deionized water, until the rinsing water became clear. A silt- and clay-sized fraction <50 μm was separated from a fraction >50 μm, which comprised stable aggregates, sand particles and particulate organic matter which was not occluded within stable aggregates (hereafter referred to as POM). The separated fractions were dried in a forced-air oven at 55 °C for 24 h.

Preliminary separation of the fraction >50 μm was performed using sodium polytungstate solutions of three densities (1.6, 1.7 and 1.8 g cm^{-3}) to determine the density for which the floating fraction contained the highest amount of organic material, determined via total

C and N analysis. The floating POM had the highest C and N concentration at a density of 1.6 g cm^{-3}, which was therefore selected for separating a POM fraction with the highest amount of organic material and the lowest contamination by mineral material, in agreement with Cerli et al. (2012).

The fraction >50 μm was consequently separated using 80 ml of sodium polytungstate solution at a density of 1.6 g cm^{-3}. To facilitate the suspension of soil particles, the container was hand-swirled and the particles remaining on the sides were washed with additional sodium polytungstate solution. The soil suspension was left undisturbed for at least 1 h, to allow the sedimentation of heavy soil particles. The floating material, composed mainly of POM, was collected and washed with deionized water until the conductivity of the rinsing solution was <50 μS cm^{-1} (Schrumpf et al. 2013). The material heavier than 1.6 g cm^{-3}, comprising stable aggregates and sand particles (hereafter referred to as stable aggregates), was washed with deionized water until the electrical conductivity of the rinsing water was <200 μS cm^{-1} (Schrumpf et al. 2013).

The fractions were dried in a forced-air oven at 55 °C for 24 h and weighed. The mass recovery was on average 99.8 % (SE=0.3 %, range 95.8–102.2 %). Recovery of C and N were 97.4 % (SE=0.5 %) and 98.4 % (SE= 0.7 %) respectively.

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Soil chemical analysis

Before chemical analysis, the soil fractions were ground to fine powder with an agate mortar. The two fractionation procedures, aggregate size fractionation and size-density fractionation, were performed at Fondazione Edmund Mach, Italy and University of Copenhagen, Denmark, respectively. Analysis of C and N via dry combustion was performed for soil fractions obtained by different fractionation procedures with different elemental analyzers. The fractions obtained through aggregate size fractionation were analyzed for C and N by dry combustion with a PerkinElmer PE2400 CHNS/O elemental analyzer (Norwalk, CT, USA), following HCl (10 %) treatment for carbonate removal in mineral soil fractions (Brodie et al. 2011). The fractions obtained through size-density fractionation were analyzed for C and N by dry combustion with a Thermo Scientific™ FLASH 2000 EA NC Analyzer (Waltham, Massachusetts, USA). As H_2SO_3 was shown to be less corrosive than HCl toward the elemental analyzer, mineral soil fractions were treated prior to dry combustion with H_2SO_3 (6 %) (Skjemstad and Baldock 2006).

The bulk soil samples were analyzed using both elemental analyzers, in order to perform calculations of C recovery for the respective fractionation procedure. Analysis of the same samples served also as a control test, to ensure that both instruments provided similar C and N concentrations. We found that C and N concentrations determined using PerkinElmer PE2400 were underestimated by 4.3 and 3.4 % respectively compared to those from Thermo Scientific™ FLASH 2000.

Calculations and statistical analysis

The C stored in the mineral soil layers was calculated according to Rodeghiero et al. (2009), using a fixed soil depth approach. This stock calculation approach was chosen as the stone content was higher than 20 % in some plots, which was shown to hamper an equivalent soil mass correction (Schrumpf et al. 2011). The soil C stocks are based on bulk soil C concentrations measured by dry combustion with a PerkinElmer PE2400 CHNS/O elemental analyzer, as presented in Guidi et al. (2014). Amounts of C stored in each fraction were calculated using the C recovery obtained following the respective fractionation procedure and dry combustion analysis.

The mean weight diameter (MWD) was calculated summing the products of the mean diameter of each aggregate size fraction and the weight percentages of the corresponding aggregate size fractions, according to Kemper and Rosenau (1986).

Statistical analyses were performed in R environment (R Development Core Team 2013). Differences within each soil depth and the 0–20 cm layer were tested with a non-parametric test (Kruskal–Wallis rank sum test) that does not assume normal distribution of the residuals. Only three replicate plots per successional stage could be analyzed, but the three replicate values were based on pooling of eight subsamples collected in each plot, thus ensuring a good coverage of the spatial variability within the three plots. After Kruskal–Wallis, a multiple comparison among successional stages was performed with the *kruskalmc* function in the R package *pgirmess* (Giraudoux 2013). Statistical significance was considered at $P<0.05$, while P values between 0.05 and 0.10 were interpreted as trends (Six et al. 2002).

The correlation between POM and root biomass was tested computing the Pearson's product–moment correlation coefficient (r) (Webster 2001).

Results

Aggregate size fractionation

In the 0–5 cm layer, earlyF and oldF showed a trend toward higher MWD than manG and abanG ($P=0.053$; Fig. 2). No significant difference in MWD was reported among successional stages in the 5–10 cm ($P=0.13$) and 10–20 cm layers ($P>0.20$; Fig. 2).

The proportion of SOC (i.e. relative distribution of SOC) in aggregate size fractions was not significantly affected by successional stages at the three depths (0–5 cm, 5–10 cm and 10–20 cm; Table 2). In the 0–5 cm layer, earlyF and oldF showed greater but not significantly different values for the SOC allocation to large macroaggregates than manG and abanG ($P=0.12$; Table 2). The SOC stock within aggregate size fractions was significantly affected by successional stages in the 0–5 cm and 5–10 cm layers, and marginally affected in the 10–20 cm layer. In the 0–5 cm and 5–10 cm layer, the amount of SOC stored in small macroaggregates ($P=0.029$ in 0–5 cm, and $P=0.053$ in 5–10 cm), microaggregates ($P=0.04$) and silt and clay ($P=0.047$ in 0–5 cm and $P=0.09$ in 5–10 cm) was significantly or
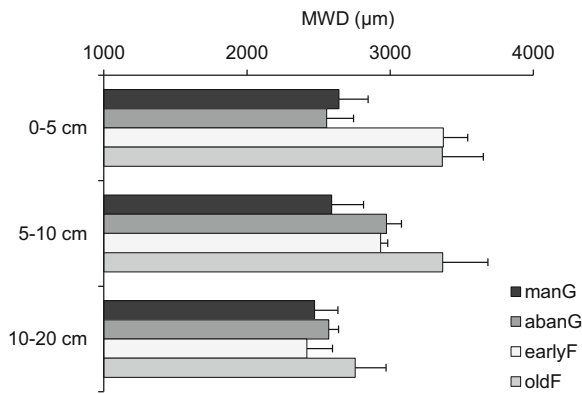
Fig. 2 Depth distribution of mean weight diameter (MWD) at the successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest). *Error bars* represent the standard error of the mean (n=3). If no letters are present, no significant differences were observed among successional stages within each depth based on multiple comparisons after Kruskal-Wallis, with P<0.05

marginally affected by successional stages, while no significant effect on large macroaggregate SOC stocks was detected (P>0.15). At 0–5 cm depth, small macroaggregates stored a greater SOC stock in abanG than in oldF (Table 2), and the amount of SOC stored in microaggregates and silt and clay decreased in the order manG ≥ abanG, earlyF ≥ oldF (Table 2). In the 5–10 cm layer, small macroaggregates and silt and clay showed a trend toward more SOC in manG and abanG than earlyF and oldF, and microaggregates stored more SOC in manG than in oldF (Table 2). In the 10–20 cm layer, the SOC stock in small macroaggregates was marginally affected by successional stages (P=0.09), with a tendency toward higher SOC stock in manG than in oldF (Table 2).

Size-density fractionation

Significantly higher mass was observed for stable aggregates from size-density fractionation (hereafter referred to as stable aggregates) in the 0–5 cm layer (P=0.033) in abanG than in oldF, and a similar trend was observed for the other layers (P=0.07 in 5–10 cm, P=0.08 in 10–20 cm; Table 3). The mass of POM fraction was marginally affected by successional stages (P=0.08 in 0–5 cm, P=0.07 in 5–10 cm and 10–20 cm), with a trend toward higher values in oldF and abanG compared with manG in the 0–5 cm layer and toward higher values in oldF compared with other successional stages in the 5–10 and 10–20 cm layer (Table 3). Significantly higher mass of the silt- and clay-sized fraction (P=0.041) was

found in oldF than in abanG, and a similar trend was also observed for the other layers (P=0.07 in 5–10 cm and P=0.08 in 10–20 cm; Table 3).

The OC concentration of size-density fractions was not significantly affected by successional stage in any of the three mineral soil layers (Table 3). However, in the 0–5 cm layer the OC concentration of the silt- and clay-sized fraction (P=0.08) was marginally affected by successional stages, with a trend toward higher concentrations in abanG than in oldF (Table 3). The C/N ratio of size-density fractions was significantly affected by successional stages, with values generally increasing from manG to oldF (Fig. 3), mirroring the trend in C/N ratio of bulk soil samples (Table 1). Moreover, the C/N ratio in POM increased with depth (P =0.007), in contrast to other size-density fractions and bulk soil samples (Fig. 3; Table 1).

The relative SOC allocation to stable aggregates was significantly affected by successional stages in the 0–5 cm layer (P=0.019) and marginally affected in the 5–10 cm and 10–20 cm layers (P=0.07 in 5–10 cm, P=0.08 in 10–20 cm; Table 4). In the 0–5 cm layer, the allocation of SOC to stable aggregates drastically decreased in the order manG ≥ abanG, earlyF ≥ oldF, with manG having significantly higher proportion of SOC than oldF (Table 4). At 5–10 cm and 10–20 cm depth, manG and abanG similarly tended to have a higher SOC proportion in stable aggregates than oldF (Table 4). The oldF stored a significantly higher fraction of SOC in POM than manG in all mineral soil layers, with intermediate values for abanG and earlyF (P=0.041 in 0–5 cm, P=0.027 in 5–10 cm and P=0.047 in 10–20 cm; Table 4). Moreover, the proportion of SOC stored in POM was significantly related to root biomass in the 0–5 cm (r=0.68; P=0.014), 5–10 cm (r=0.54; P=0.027) and 10–20 cm layer (r=0.69; P=0.013). The proportion of SOC stored in the silt- and clay-sized fraction was marginally affected by successional stages in the three mineral soil layers (P≥0.05; Table 4), with a trend toward lower SOC allocation to the silt- and clay-sized fraction in abanG than in manG and oldF in the 0–5 cm and 10–20 cm layer.

The SOC stock in stable aggregates was affected by successional stages in all mineral soil layers (P=0.016 in 0–5 cm, P=0.024 in 5–10 cm, P=0.043 in 10–20 cm; Table 4). In the 0–5 cm layer, the SOC stock in stable aggregates decreased in the order manG ≥ abanG, earlyF ≥ oldF, with significantly higher SOC stocks in manG than in oldF (Table 4). In the 5–10 cm and 10–

Table content:

Table 2 Proportion of SOC and SOC stock in aggregate size fractions at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest), in 0–5, 5–10 and 10–20 cm

		Proportion of SOC (%)				SOC stock (Mg ha^{-1})			
		Large macroaggregates	Small macroaggregates	Microaggregates	Silt and clay	Large macroaggregates	Small macroaggregates	Microaggregates	Silt and clay
0–5 cm	manG	42.5 (2.7)	42.1 (2.7)	12.1 (3.6)	3.3 (0.1)	11.2 (1.5)	11.0 (0.9)ab	3.1 (0.8)a	0.9 (0.0)a
	abanG	32.8 (5.8)	54.8 (6.3)	10.2 (1.1)	2.2 (0.3)	7.4 (1.6)	12.1 (1.1)a	2.3 (0.3)ab	0.5 (0.1)ab
	earlyF	55.3 (5.3)	37.0 (3.8)	6.3 (1.4)	1.4 (0.2)	8.9 (0.8)	6.0 (0.7)ab	1.0 (0.2)ab	0.2 (0.0)b
	oldF	54.4 (8.4)	34.8 (6.5)	8.0 (0.9)	2.8 (1.0)	7.8 (1.5)	4.9 (0.9)b	1.1 (0.1)b	0.4 (0.1)ab
5–10 cm	manG	43.0 (3.5)	39.9 (2.0)	12.6 (4.2)	4.5 (0.5)	8.4 (0.9)	7.8 (0.5)	2.4 (0.7)a	0.9 (0.1)
	abanG	50.6 (3.1)	40.2 (3.8)	6.7 (0.2)	2.5 (0.6)	9.7 (0.5)	7.7 (0.8)	1.3 (0.1)ab	0.5 (0.1)
	earlyF	48.1 (2.0)	42.4 (1.2)	7.4 (0.7)	2.1 (0.3)	7.1 (0.5)	6.3 (0.0)	1.1 (0.1)ab	0.3 (0.0)
	oldF	59.2 (8.6)	30.5 (6.1)	7.1 (1.5)	3.1 (1.2)	7.9 (1.2)	4.1 (0.8)	0.9 (0.2)b	0.4 (0.2)
10–20 cm	manG	40.8 (2.9)	41.7 (2.3)	12.1 (2.7)	5.4 (0.8)	10.7 (1.4)	10.8 (0.4)	3.2 (0.7)	1.4 (0.1)
	abanG	40.8 (3.1)	45.9 (3.6)	9.9 (1.2)	3.5 (0.4)	9.3 (1.1)	10.6 (1.8)	2.2 (0.1)	0.8 (0.0)
	earlyF	37.8 (3.1)	50.1 (3.0)	9.0 (0.3)	3.1 (0.4)	8.4 (1.0)	11.0 (0.6)	2.0 (0.2)	0.7 (0.1)
	oldF	47.5 (6.9)	35.6 (4.6)	11.1 (0.8)	5.7 (1.8)	8.9 (1.2)	6.7 (0.9)	2.1 (0.2)	1.1 (0.3)

Values are means ($n=3$) with standard error of the mean in brackets. Within each aggregate size fraction and depth, different letters indicate significant differences based on multiple comparisons following Kruskal-Wallis, with $P<0.05$. If no letters are present, no significant differences were observed among successional stages
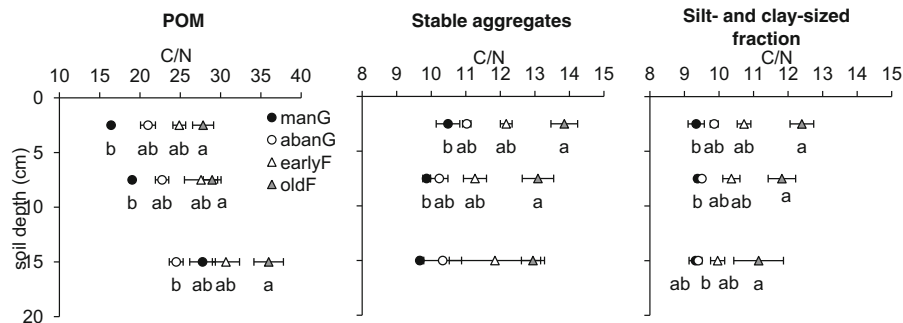
Table 3 Mass and OC concentration in size-density fractions at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest), in 0–5, 5–10 and 10–20 cm

		Mass (%)			OC concentration (mg g^{-1})		
		POM	Stable aggregates	Silt- and clay-sized fraction	POM	Stable aggregates	Silt- and clay-sized fraction
0–5 cm	manG	1.8 (0.0)	81.5 (2.0)ab	16.8 (2.0)ab	318 (12)	78 (3)	58 (3)
	abanG	4.2 (0.4)	84.1 (0.4)a	11.7 (0.1)b	288 (18)	78 (2)	67 (4)
	earlyF	3.3 (0.4)	75.7 (1.4)ab	21.0 (1.3)ab	338 (8)	68 (6)	54 (5)
	oldF	3.8 (1.0)	72.4 (2.2)b	23.8 (2.8)a	342 (16)	50 (12)	46 (7)
5–10 cm	manG	0.6 (0.1)	77.4 (4.0)	22.0 (4.1)	303 (6)	45 (2)	43 (2)
	abanG	1.3 (0.2)	83.7 (0.5)	15.0 (0.4)	275 (16)	52 (1)	52 (1)
	earlyF	1.4 (0.1)	74.2 (1.5)	24.4 (1.4)	314 (22)	47 (7)	45 (6)
	oldF	2.1 (0.4)	67.9 (2.8)	30.1 (3.0)	318 (11)	39 (11)	39 (8)
10–20 cm	manG	0.3 (0.0)	64.2 (1.1)	35.5 (1.1)	323 (34)	23 (2)	27 (3)
	abanG	0.7 (0.2)	76.0 (0.6)	23.4 (0.8)	255 (13)	35 (1)	36 (1)
	earlyF	0.6 (0.1)	67.2 (2.0)	32.2 (2.1)	354 (13)	32 (4)	32 (2)
	oldF	1.1 (0.2)	62.8 (4.9)	36.1 (5.1)	355 (14)	31 (11)	34 (10)

Values are means ($n=3$) with standard error of the mean in brackets. Within each size-density fraction and depth, different letters indicate significant differences based on multiple comparisons following Kruskal-Wallis, with $P<0.05$. If no letters are present, no significant differences were observed among successional stages

20 cm layer, stable aggregates in abanG stored more SOC than in oldF (Table 4). In all mineral soil layers, the SOC stored in POM showed a trend toward greater values in oldF than in manG and abanG ($P=0.08$ in 0–5 cm and 5–10 cm, $P=0.09$ in 10–20 cm; Table 4). The SOC stored in the silt- and clay-sized fraction did not differ significantly among successional stages in 0–5 cm and 5–10 cm layers ($P>0.20$; Table 4), whereas the silt- and clay-sized fraction showed a trend toward less SOC in abanG than in manG in the 10–20 cm layer ($P=0.06$; Table 4).

The SOC stored in the size-density fractions was calculated for the 0–20 cm layer (Fig. 4). The decrease in mineral SOC stock from manG to oldF in this layer, as shown by Guidi et al. (2014), mainly took place in stable aggregates ($P=0.022$), whereas POM-C tended to be higher in oldF than in manG ($P=0.06$; Fig. 4). The total SOC stocks, including the organic layers and the 0–20 cm layer of mineral soil, did not differ significantly among successional stages but the SOC distribution among physical SOM fractions changed following forest expansion on grassland. The POM and organic

Fig. 3 Depth distribution of C/N ratio in size-density fractions at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest). *Error bars* represent the standard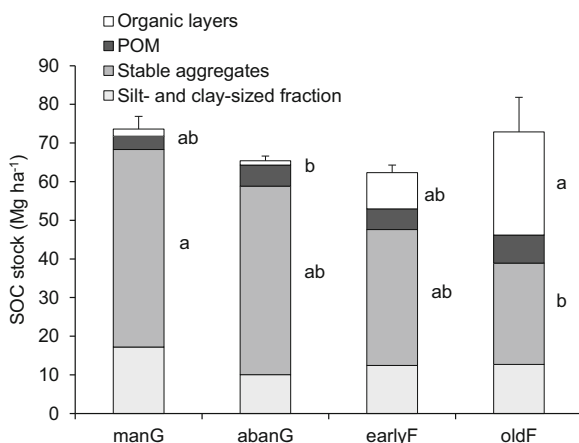 error of the mean ($n=3$). Within each depth, *different letters* indicate significant differences based on multiple comparisons after Kruskal-Wallis, with $P<0.05$. If no letters are present, no significant differences were observed among successional stages

Table 4 Proportion of SOC and SOC stock in size-density fractions at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest), in 0–5, 5–10 and 10–20 cm

Depth	Stage	Proportion of SOC (%)			SOC stock (Mg ha^{-1})		
		POM	Stable aggregates	Silt- and clay-sized fraction	POM	Stable aggregates	Silt- and clay-sized fraction
0–5 cm	manG	7.1 (0.4)b	80.6 (1.2)a	12.3 (1.5)	1.8 (0.0)	21.1 (1.2)a	3.3 (0.6)
	abanG	14.3 (1.7)ab	76.5 (1.9)ab	9.2 (0.1)	3.2 (0.5)	17.0 (0.6)ab	2.0 (0.1)
	earlyF	15.2 (2.1)ab	69.6 (1.9)ab	15.2 (1.5)	2.5 (0.4)	11.2 (0.4)ab	2.4 (0.2)
	oldF	21.5 (2.9)a	59.3 (3.3)b	19.1 (4.0)	3.1 (0.5)	8.4 (0.9)b	2.6 (0.4)
5–10 cm	manG	3.7 (0.8)b	75.1 (4.9)	21.1 (5.4)	0.7 (0.2)	14.7 (0.9)ab	4.1 (1.1)
	abanG	6.6 (1.3)ab	79.3 (1.4)	14.2 (0.2)	1.3 (0.3)	15.2 (0.3)a	2.7 (0.0)
	earlyF	9.1 (1.7)ab	69.0 (3.0)	21.9 (1.4)	1.3 (0.3)	10.2 (0.4)ab	3.2 (0.3)
	oldF	14.9 (1.5)a	58.0 (4.6)	27.1 (4.0)	2.0 (0.3)	7.7 (0.8)b	3.6 (0.4)
10–20 cm	manG	3.3 (0.2)b	59.3 (2.4)	37.4 (2.2)	0.9 (0.1)	15.4 (0.6)ab	9.8 (1.1)
	abanG	4.5 (1.1)ab	72.7 (0.7)	22.8 (1.6)	1.0 (0.2)	16.6 (1.6)a	5.3 (0.9)
	earlyF	7.1 (1.6)ab	62.4 (0.8)	30.5 (1.3)	1.6 (0.4)	13.7 (0.6)ab	6.7 (0.4)
	oldF	11.7 (1.7)a	53.4 (7.3)	34.9 (6.2)	2.2 (0.3)	10.0 (1.5)b	6.5 (1.0)

Values are means ($n=3$) with standard error of the mean in brackets. Within each size-density fraction and depth, different letters indicate significant differences based on multiple comparisons following Kruskal-Wallis, with $P<0.05$. If no letters are present, no significant differences were observed among successional stages

layers contributed together to 7 and 10 % of SOC stocks in manG and abanG respectively, whereas their contribution increased to 26 and 47 % in earlyF and oldF.

Fig. 4 SOC stocks at the four successional stages (manG: managed grassland; abanG: abandoned grassland; earlyF: early-stage forest; oldF: old forest) in size-density fractions within the mineral soil (0–20 cm) and in organic layers. *Error bars* represent the standard error of the mean of total SOC stocks ($n=3$). *Different letters* indicate significant differences within fractions based on multiple comparisons after Kruskal-Wallis, with $P<0.05$. If no letters are present, no significant differences were observed among successional stages

Discussion

Changes in aggregate size and stability

Following forest expansion on abandoned grassland in the study area, the MWD of aggregate size fractions tended to increase in the 0–5 cm layer. The decrease in mineral SOC stocks reported after forest expansion on grassland in the study area (Guidi et al. 2014) took place mainly in aggregate size classes <2,000 μm, whereas the SOC stock in large macroaggregates did not change significantly following LUC. Differences in aggregate size fractions were more evident in upper soil layers (e.g. 0–5 cm layer), in agreement with findings that deeper layers are affected to a lesser extent by changes in litter input, which prevents detection of significant changes (Poeplau and Don 2013).

Only a few studies explored changes in physical SOM fractions following conversion from grassland to forest and, to our knowledge, this is the first study that explores changes in soil aggregation following natural forest expansion on abandoned grasslands in the European Alps. While John et al. (2005) found no significant difference in MWD between forest and grassland sites, Liao et al. (2006) observed an increased C association with macroaggregates after woody plant

invasion of grasslands. Our results similarly showed that the mean dimension of aggregates tended to increase following natural forest succession in the investigated area. Soil aggregation often relates positively with SOC content (Six et al. 1998) but we found that grassland plots, which had higher SOC stocks than forest plots, mainly stored C in small rather than in large macroaggregates. This pattern could be related to differences in root morphology and turnover, which can affect both aggregate size and SOC storage (Jastrow et al. 1998). Grasses generally have smaller fine root diameter than trees (on average 0.22 vs 0.58 mm; Jackson et al. 1997) which can contribute to increase SOC storage in small macroaggregates (Jastrow et al. 1998). Moreover, as fungal hyphae lead to the formation of large macroaggregates by physical enmeshment and exudation of binding agents (Tisdall 1991), a shift from a bacterial- to a fungal-dominated microbial community (Macdonald et al. 2009) can contribute to increase the formation of larger aggregates following grassland abandonment and forest development.

Although the dimension of aggregates separated by aggregate size fractionation tended to increase with forest expansion, our results showed a decrease in the relative SOC allocation and storage in stable aggregates from size-density fractionation following forest expansion in the study area. While aggregate size fractionation separated soil aggregates through wet-sieving, size-density fractionation separated aggregates resistant toward more pronounced physical disturbance (Magid et al. 1996, 2002; Christensen 2001; Zimmermann et al. 2007), thus explaining the apparently contrasting results obtained following the two procedures. However, similarly to the results in aggregate size fractions, differences in mass and SOC allocation to stable aggregates among successional stages were more evident in the 0–5 cm layer, thus confirming that upper soil layers are more sensitive to changes in aggregate size and stability than deeper ones.

Considering that aggregate stability followed a gradual and consistent trend across successional stages and that the main soil properties influencing aggregation (e.g. soil texture, inorganic C content) were similar across successional stages, we interpret the decreased aggregate stability as the result of natural succession from grassland to forest in the study area. The decrease in aggregate stability confirmed our expectations and is in agreement with findings of reduced SOC storage in stable aggregates following afforestation of six

grassland sites across Europe, three of which were located in alpine and pre-alpine regions (Poeplau and Don 2013).

Differences in the ecology of grasslands vs forests could have led to the observed changes in stable aggregates obtained by size-density fractionation. Lower mass, length and turnover of fine roots in forests compared with grasslands (Guo et al. 2007) can lead to reduced aggregate stability, as fine roots exert a positive effect on aggregate stabilization (Jastrow et al. 1998). More recalcitrant root C input, as that produced by trees compared with grasses (e.g. higher C/N ratio and lignin content) (Hiltbrunner et al. 2013) can lead to lower microbial substrate use efficiency, and thus to reduced aggregate stability (Cotrufo et al. 2013). Moreover, ceased manure application can contribute to lower aggregate stability following grassland abandonment, considering that manure application can have enhanced microbial activity and thus aggregate stability in the managed grassland (Aoyama et al. 2000). As soil warming can promote aggregate stability through physico-chemical processes and through the enhancement of soil biological activity (Dimoyiannis 2009), reduced solar radiation and soil temperature especially during the growing season (Nosetto et al. 2005; Hiltbrunner et al. 2013) can also have contributed to decrease the formation of stable aggregates following forest expansion on former grasslands in the study area.

Changes in the POM fraction

A larger proportion of SOC was allocated to POM in oldF than in manG, and oldF stored 3.8 Mg ha^{-1} more POM-C than manG in the 0–20 cm layer. Despite a trend toward increased POM-C following succession from grassland to forest, the transitional phases (abanG, earlyF) were not significantly different from one other and from the long-term land uses (manG, oldF).

Differences in SOC allocation to POM were mainly driven by differences in mass among successional stages, and to lesser extent by differences in POM-C concentration, which did not differ significantly among successional stages. The higher C allocation to POM can therefore be attributed to higher POM accumulation rather than to intrinsic differences in its C concentration. The higher SOC allocation to POM in oldF than in manG is in agreement with results reported by John et al. (2005), who found higher SOC allocation to

POM in a Norway spruce forest than in a grassland site. Under our study conditions, higher quality of OM input (e.g. high N and low lignin) in grassland than in forest sites could have led to higher microbial activity in the grasslands (Knorr et al. 2005) and in turn to lower POM accumulation. As POM is mainly composed of uncomplexed OM derived from recently incorporated residues (Gregorich et al. 2006), the lower C/N ratio in POM of manG than in oldF can be considered as an indicator of higher quality of OM input, owing to differences in litter input and to annual application of manure. In contrast to other size-density fractions and bulk soil, the C/N ratio of POM increased with depth. This can be explained by lower N content of roots in deeper layers (Genenger et al. 2003; Ugawa et al. 2010) but also to a lower degree of OM transformation, attributed by Budge et al. (2011) to physical and nutritional constraints that limited biological activity in deeper layers of alpine grasslands.

As root litter constitutes a significant proportion of POM (Baisden et al. 2002; Dalal et al. 2005), slower decay of root-derived OM inputs as a result of differences in root chemistry and the prevalence of coarse roots in forest (Silver and Miya 2001; Guo et al. 2008) could have contributed to increase POM accumulation in oldF than in manG. This is supported by the positive and significant correlation found between root biomass and the proportion of SOC in the POM fraction. Moreover, decreased soil temperature and lower moisture following forest succession (Hiltbrunner et al. 2013) could have further contributed to increase POM accumulation, through reduction of microbial decay rates (Six et al. 1998; Creamer et al. 2011).

The allocation of C to the POM fraction increased gradually with succession from grassland to forest; however, transitional phases (abanG, earlyF) were not significantly different from long-term land uses (manG, oldF). Following subalpine grassland abandonment, Meyer et al. (2012) showed an increased proportion of POM-C, which was attributed to increased litter input and decreased litter quality (i.e. high C/N and low nutrient concentrations). Reduced grassland management, e.g. shift from mowing to grazing, was also shown to increase POM-C according to Leifeld and Fuhrer (2009). Contrasting results were previously reported for responses of POM-C to forest growth on former grasslands. Clark et al. (2012) studied chronosequences of hardwood forests in western New England and reported that the proportion of POM-C decreased by 16–

40 % after 25 and 40 years following forest establishment on abandoned meadows and pastures, whereas Liao et al. (2006) observed POM-C accumulation following woody encroachment into grasslands. In our study, the increase in POM-C was detectable only between long-term land uses (manG, oldF). This could have been due to gradual changes in decomposition rates of POM following natural succession, as environmental conditions and soil decomposer community (Smith and Johnson 2004; Macdonald et al. 2009) could have gradually and progressively changed from abanG to oldF.

The increase in POM-C from manG to oldF only partly compensated the SOC decrease in stable aggregates from size-density fractionation, leading to an overall decrease in mineral SOC stocks (0–20 cm) and to unchanged total SOC stocks when organic layers were included (Guidi et al. 2014). The increased contribution of organic layers and POM to total SOC stocks suggests that the physical SOC stability decreased following natural succession from grassland to forest. The C stored in the organic layers responds faster to environmental and management modifications than C in mineral soil layers (Currie 1999). As well as C in the organic layers, POM-C can undergo rapid losses when ecosystems are disturbed (Cambardella and Elliott 1992; Dalal et al. 2005), although in some cases POM can retain substantial C amounts following disturbances (Gregorich et al. 2006). The altered C distribution among soil compartments in our study area therefore suggests that SOC can become more vulnerable to changes in environmental and management conditions following succession from grassland to forest.

Conclusions

To our knowledge, this is the first study that investigated changes in SOC distribution within physical SOM fractions following natural forest expansion on abandoned grassland in the European Alps. The combination of two physical SOC fractionation procedures showed that the dimension of aggregates separated by aggregate size fractionation increased following conversion from grassland to forest while stable aggregates from size-density fractionation stored a lower relative and absolute SOC amount in forest than in grassland plots. The decrease in mineral SOC stock (0–20 cm) following forest expansion on former grasslands mainly took place

in stable aggregates, while POM-C tended to increase from manG to oldF, most likely due to differences in the quality of OM input, microclimatic conditions and decomposer community. The decreased SOC stock in stable aggregates and increased POM-C stocks together with the higher contribution of organic layers to total SOC stocks suggest that the physical protection of SOC declined during natural succession from grassland to forest, thus leading to higher SOC susceptibility to external disturbances such as forest management and environmental change.

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