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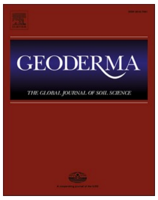

# Variations in soil aggregation, microbial community structure and soil organic matter cycling associated to long-term afforestation and woody encroachment in a Mediterranean alpine ecotone

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## ABSTRACT

Afforestation and subsequent expansion of trees on former grasslands may significantly impact the structure and activity of the soil microbial community, altering soil aggregation and affect its potential to store and cycle organic matter (OM). We investigated OM dynamics in aggregate-size topsoil samples collected along a Mediterranean alpine ecotone consisting of three vegetation types (grassland/shrubland, mixed shrubland-pine, and pine forest) in central Spain. Analytical determinations of soil organic carbon (SOC), total nitrogen (TN), particulate OM (POM), mineral-associated OM (MaOM), and the stable isotopic composition of carbon were conducted in each of the four aggregate-size fractions considered. Additionally, the structure of the microbial community (assessed as PLFA abundance), and the β-glucosidase and β-glucosaminidase activities were determined in bulk soil samples. More than half of the soil mass was contained within small macroaggregates regardless of vegetation type. SOC and TN values increased with decreasing aggregate-size classes across all vegetation types. The stability of microaggregates was negatively affected by the expansion of woody vegetation, which resulted in tree-dominated stands showing comparatively lower SOC and TN values in the smaller aggregate-size classes. On the other hand, these vegetation dynamics promoted soil macro-aggregation. While SOC contents did not show significant differences between land covers, vegetation shifts induced changes in the soil microbial community. Soil $\delta^{13}C$ values, the abundance of gram-positive bacteria and β-glucosidase activity were significantly higher in grasslands/shrublands than in forests, while significantly higher fungi/bacteria ratio was observed in forests. Small macroaggregates appear to play a key role in the stabilisation of relatively unprocessed OM across all vegetation types, as suggested by their significantly higher concentrations of POM. However, this fraction represents the most labile pool of OM, and as such, it is the most exposed to mineralisation. We conclude that the afforestation and potential vegetation shifts experienced in Mediterranean alpine grasslands lead to distinct changes in soil microbial communities, aggregation and soil OM dynamics, which given the strong temperature sensitivity to decomposition commonly reported in cold environments, suggests that soil OM in these high-elevation ecosystems may become highly vulnerable to environmental change.

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*Abbreviations:* SL, grassland/shrubland; SL-FO, shrubland/forest; FO, forest; SOC, soil organic carbon; LMa, large macroaggregates (6–2 mm); SMa, small macroaggregates (2–0.250 mm); Mi, microaggregates (0.250–0.053 mm); s+c, silt + clay size particles (<0.053 mm); MWD, mean weight diameter; MGD, mean geometric diameter; POM, particulate organic matter; MaOM, mineral-associated organic matter; NAGase, *N*-acetyl-β-glucosaminidase; PLFA, phospholipid fatty acid; AMF, arbuscular mycorrhizal fungi; Gram+, gram-positive bacteria; Gram-, gram-negative bacteria; RDA, redundancy analysis.

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## 1. Introduction

Afforestation of Mediterranean alpine grasslands has been taking place for over half a century as a result of various factors including economic constraints negatively impacting rural livelihoods. These conditions have caused the progressive migration of rural populations to urban settlements and the nearly disappearance of extensive livestock practices, which have fuelled government-sponsored afforestation programmes in these sparsely populated regions (Ortiz et al., 2016). In a similar vein, there has also been a continuous expansion of woody vegetation at a global scale in recent decades, that has been mainly driven by increasing concentrations of atmospheric $CO_2$ (Donohue et al., 2013), and the abandonment of agricultural land (Smal and Olszewska, 2008). This phenomenon, also referred to as woody encroachment, is particularly marked in Mediterranean alpine regions, where grassland areas get progressively transformed into shrub-dominated ecosystems, prior to trees becoming established (Chauchard et al., 2010; Leonelli et al., 2011). Such shifts in vegetation cover cause changes in carbon (C) cycling and $CO_2$ sequestration through the accumulation of C in the woody biomass (Archer et al., 2001; Kammer et al., 2009). Afforestation and woody vegetation encroachment may also alter the storage and residence time of soil organic matter (SOM) due to variations in the quality and quantity of litter entering the soil, and associated changes in edaphic properties that affect the function, structure and activity of the soil microbial community (Kuzyakov, 2006; von Lützow et al., 2006; Curiel Yuste et al., 2012; Guidi et al., 2014a; Saiz et al., 2015).

Soil aggregation processes include the mechanisms involved in binding soil particles into stable aggregates, which are the basic units of soil structure, and which strongly influence SOM dynamics and soil biodiversity (Oades, 1984). The formation of soil aggregates is strongly related to the quality and quantity of SOM (Oades, 1984; Six et al., 1998). Tisdall and Oades (1982) have long demonstrated that soil microaggregates are bound together within macroaggregates by transient and temporary binding agents, whereby new microaggregates are formed around particulate OM (POM) material inside macroaggregates when OM fragments become colonized by microorganisms and are encrusted by mineral materials (Oades, 1984; Gale et al., 2000). This aggregate hierarchy and the crucial role that OM plays in soil aggregation are commonly reported for temperate soils (Six et al., 2004). Aggregates protect SOM through the formation of physical barriers between the substrate and the microbial biomass, thus limiting enzymatic and microbial decomposition processes. At the same time, soil microbiota have positive effects on the development of soil structure, as they contribute to the formation of aggregates through the binding of mineral particles and OM (Six et al., 2004). Biotic-mediated soil aggregation is mainly promoted by fungal hyphae, which foster the mechanical union of soil particles, and through exudation of by-products resulting in the union of primary particles (Six et al., 2004; Garcia-Franco et al., 2015). Within this context, fungi have been shown to be more important than bacteria in promoting soil aggregation (De Gryze et al., 2005).

Several studies have investigated how afforestation (Hiltbrunner et al., 2013; Wei et al., 2013a; Garcia-Franco et al., 2015), deforestation (Wei et al., 2013b), and vegetation shifts (Guidi et al., 2014b; Saiz et al., 2015) influence the role of aggregates in protecting SOM. While the conversion of agricultural land to forest can increase soil organic carbon (SOC) levels due to the accumulation of C in macroaggregates and its redistribution from fine particles to coarser fractions (Wei et al., 2013a), the effect of tree establishment on SOC and aggregate dynamics is less univocal in grasslands (Berthrong et al., 2012; Guo et al., 2007; Saiz et al., 2015). In addition to direct biotic effects, afforestation and woody encroachment may affect soil aggregation through the alteration of soil microclimatic conditions caused by the presence of trees (i.e. provision of shade, soil temperature insulation, etc.; Hagedorn et al., 2014). Given the complexity of the mechanisms involved in soil physical aggregation, there is a need for a more in-depth exploration of the dynamics of aggregate formation that occur specifically in Mediterranean alpine regions, where an alteration in OM occluded within aggregates can be expected due to forest encroachment caused by global environmental change. To better address these research needs, aggregate-size class analyses can be used in combination with the natural abundance of stable C isotopes to study SOM dynamics as affected by vegetation shifts and/or changes in land use (Saiz et al., 2015; Saiz et al., 2016; Ortiz et al., 2016; Gerschlauer et al., 2019). Besides serving as a good proxy for the degree of mineralization of SOM, another obvious advantage of using the $\delta^{13}C$ of SOM in biogeochemical research is that this pool integrates the isotopic signature of the precursor biomass over various spatio-temporal scales.

Significant variations in vegetation cover may induce changes in soil microbial activity by quantitative effects on the amount of aboveground litter and root exudates (Renella et al., 2007), but also by qualitative differences in the substrate available for microbial decomposition (Fernández-Alonso et al., 2018). These vegetations shifts may also affect the structure of the soil microbial community, whose composition can be assessed with analyses of phospholipid fatty acids (PLFAs). The use of specific PLFA markers provides relevant information about the concentration of bacterial communities, protozoan, actinomycetes as well as saprophytic and arbuscular mycorrhizal fungi. Additionally, the potential extracellular activities of the β-glucosidase and the N-acetyl-β-glucosaminidase (NAGase) enzymes offer valuable insights about the microbial degradation of key organic compounds. The β-glucosidase activity in soils is directly related to OM cycling as it catalyses the final step of cellulose degradation that produces glucose, an essential energy source in soil environments (Sotomayor-Ramírez et al., 2009). The NAGase enzyme plays a central role in the degradation of chitin, a primary compound of the cell walls of fungi and the exoskeletons of arthropods, while β-glucosidase is pivotal in cellulose degradation (Sinsabaugh, 2005).

The present study aims to investigate the long-term effects of afforestation and subsequent tree encroachment on the storage and potential stabilization of SOM in a grassland-shrubland Mediterranean alpine ecosystem. The specific objectives of this work were to evaluate the effect of vegetation shifts in (1) soil aggregation patterns, (2) organic carbon (OC) and total nitrogen (N) of soil aggregate-size classes, and (3) soil microbial community. We have chosen to work on topsoil samples as SOM variations caused by changes in land cover are typically more evident in the most superficial soil layers (Poeplau and Don, 2013; Guidi et al., 2014a;b). Our first working hypothesis was that macroaggregates are more abundant in forests and that their mean aggregate size is higher than in grassland-shrubland soils. Our second hypothesis was that aggregate-associated SOC increases with aggregate size. Our third hypothesis stated that grassland/shrubland soils present higher $\delta^{13}C$ values than those of forests. Finally, we hypothesized that composition and activity of microbial communities vary across the different vegetation covers. We sampled soils from three sites corresponding to grassland/shrubland, mixed shrubland-pine vegetation and pine forest plots. We determined soil aggregate-size class distribution and conducted a SOM fractionation scheme. Total OC and N concentrations, as well as the stable C isotopic composition were obtained for each size fraction. Additionally, we determined the structure of the soil microbial community and quantified β-glucosidase and NAGase enzyme activities on bulk samples. These data could prove very useful in advancing our understanding in SOM dynamics caused by vegetation shifts driven by global change in Mediterranean alpine environments.

## 2. Materials and methods

### 2.1. Study area

The study area is located on the northern face of the Sierra de Guadarrama in the Sistema Central mountainous range in Spain (40° 59′ N; 3° 49′ W) at an altitude between 1900 and 2000 m a.s.l. The Sistema

Central is characterised by a sub-Mediterranean climate (Walter, 1985) with high year-round temperature fluctuations, snow cover on high-elevation areas throughout the winter, and warm and dry summers (García Romero and Muñoz Jiménez, 2010). Mean annual temperature is 5.2 °C, mean minimum temperature of the coldest month is −3.8 °C, mean maximum temperature of the hottest month is 21.7 °C, and mean annual precipitation is 1609 mm (Sánchez Palomares et al., 1999). Soils in the area are developed on granitic bedrock and they are characteristically shallow, which provided further justification to focus our work on topsoil samples. These soils are classified as Dystric Cambisol (IUSS Working Group WRB, 2014). The main characteristics of the soils are shown in Table 1.

### 2.2. Experimental setup

Our study was conducted in an ecotone area encompassing an alpine grassland/shrubland ecosystem and a Scots pine (*Pinus sylvestris* L.) forest plantation. Vegetation dynamics in the area were identified using aerial orthophotographs from 1965 and 2009. Images revealed that afforestation had occurred at least 40 years prior to soil sampling, and grasslands were still present in the vicinity under the same soil type and position within the landscape. This time span is sufficient to ensure that direct short-term effects on SOM dynamics caused by planting operations have fundamentally subsided (Poeplau and Don, 2013), thus allowing us to assess SOM changes due to vegetation shifts in the longer run. Grazing is virtually non-existent nowadays; having <0.25 livestock units per ha, and only takes place for a few weeks in summer.

Three different vegetation types were identified in the study area, namely:

i) a grassland/shrubland area (denoted hereafter as SL) mainly composed by patches of *Cytisus oromediterraneus* Rivas Martínez and *Juniperus communis* L., with *Festuca curvifolia* Lag. ex Lange, and *Luzula lactea* (Link.) E. Mey, occupying the areas between patches and with a minimal presence of *P. sylvestris* seedlings;

ii) an area of shrubland and sparse Scots pine forest (denoted hereafter as SL-FO) comprising patches of *C. oromediterraneus*, *J. communis* and *P. sylvestris* with low tree density (560 trees ha$^{-1}$), and low mean height (3.5 m), with a discontinuous, shallow forest floor. This vegetation type resulted from the natural establishment of tree seedlings originating from the neighbouring Scots pine plantation, which serves to introduce the third vegetation type;

iii) a Scots pine (*P. sylvestris*) forest area (denoted hereafter as FO) having a tree density of 1,200 trees ha$^{-1}$, a mean height of 8.9 m, and a mean forest floor thickness of 4.3 ± 2.4 cm.

Three replicated transects (20 m × 150 m) were established along the three vegetation types. These were separated by 5 m, covering a total area of 70 m × 150 m. Each transect contained three plots (20 m × 50 m) established at each of the three vegetation types. This case study was carried out within a constricted area to ensure comparable geomorphological characteristics for the selected sites. It is worth noting that the objective of the study is not to calculate SOM budgets at the regional level, as this would certainly require a far greater spatially intensive sampling strategy, but to get a deeper understanding of the long-term effects caused by afforestation and subsequent tree encroachment on the storage and potential stabilization of SOM in this Mediterranean alpine ecosystem.

### 2.3. Soil sampling and processing

In June 2012, composite soil samples from the top mineral soil (0–5 cm), each comprising five random subsamples, were collected from each land cover at each plot. If present, as it was the case of FO, forest floor was removed prior to soil sampling. Soil samples were collected using stainless steel cylinders (5 cm in height and 5 cm in diameter). Additionally, two intact soil samples were taken for bulk density determination from each land cover at each plot.

The samples were placed in a portable cooler immediately after soil collection, and were transferred to the laboratory where visible roots were manually removed. Soil aliquots for determining the PLFA profile (Stenberg et al., 1998) and enzymatic activity were sieved to 2 mm and stored at −20 °C in plastic bags until analyses. An aliquot of each sample was air-dried for aggregate size studies and SOM fractionation. Samples collected at each plot were bulked together to create a single composite sample per land cover. Soil cores collected for bulk density determination were oven-dried at 105 °C until constant weight.

### 2.4. Aggregate-size class analysis

The moisture content of all samples on collection was <6% by weight, ensuring limited mechanical stress and/or aggregate breakdown during manipulation of samples in the laboratory. Recognisable plant residues and stones were removed manually before samples were passed through a 6-mm mesh sieve. Aggregate-size fractions were obtained using a dry-sieving method described in Nie et al. (2014). Briefly, 100 g of soil were sieved through nested sieves (2, 0.25 and 0.053 mm mesh sizes) mounted on a mechanical sieve shaker OASS 203 (Orto Alresa, Madrid, Spain). Three-minute sieving intervals were found to be enough to separate the aggregate-size classes while minimizing aggregate abrasion. The resulting aggregate-size classes were: large macroaggregates (LMa, 6–2 mm); small macroaggregates (SMa, 2–0.250 mm); microaggregates (Mi, 0.250–0.053 mm) and silt + clay size particles (s + c, <0.053 mm).

To assess the changes in soil structure due to afforestation, we used two aggregation indices described by Kemper and Rosenau (1986). These are the mean weight diameter (MWD) and the mean geometric diameter (MGD).

$$MWD(mm) = \sum_{i=1}^{n} x_i \times w_i \tag{1}$$

$$MGD(mm) = exp\left[\frac{\sum_{i=1}^{n} w_i \times lnx_i}{\sum_{i=1}^{n} w_i}\right] \tag{2}$$

where $w_i$ is the weight fraction (g 100 g$^{-1}$) and $x_i$ is the mean diameter of each aggregate size class (mm).

### 2.5. Soil organic matter fractionation

Sample aliquots of each aggregate size obtained were further fractionated into two particle sizes: particulate organic matter (POM) with a size over 53 μm, and mineral-associated organic matter (MaOM) with a size lower than 53 μm. Briefly, 30 g of each aggregate-size class (except s + c particles) was added to 150 ml of distilled water and allowed to stand for 12 h prior to ultrasonic dispersion. The aggregates were

**Table 1**
Main soil properties for the topsoil (0–5 cm) in the three land covers studied. SL, Grassland/Shrubland; SL-FO, Shrubland-Forest; FO, Forest.

| Land use | pH | | EC | GC (%) | Texture (%) | | | BD (g cm$^{-3}$) | Textural class |
|---|---|---|---|---|---|---|---|---|---|
| | $H_2O$ | KCl | | | Sand | Silt | Clay | | |
| SL | 4.8 | 3.6 | 36.9 | 35.0 | 70.5 | 18.1 | 11.4 | 0.98 ± 0.11 | Sandy Loam |
| SH-FO | 4.8 | 3.6 | 41.5 | 39.1 | 69.3 | 16.1 | 14.6 | 0.77 ± 0.03 | Sandy Loam |
| FO | 4.7 | 3.5 | 39.3 | 38.0 | 72.9 | 11.4 | 15.7 | 0.78 ± 0.11 | Sandy Loam |

EC: Electrical conductivity (μS cm$^{-1}$); GC: gravel content (>2 mm); BD: bulk density values are means ± standard deviation.

subsequently dispersed with an Analog 450 probe-type sonicator (Branson Sonifier, Branson Sonic Power Co., Danbury, CT, USA) with a 125-mm diameter flat tip immersed in the solution to a depth of 15 mm. An output energy of 250 J ml$^{-1}$ was deemed sufficient to completely disperse the samples without significantly redistributing the SOM between particle size fractions (Schmidt et al., 1999). The solution was continuously stirred to facilitate the ultrasonic effect and cooled in a water bath to prevent any significant increase in temperature that might affect SOM properties (Kaiser and Berhe, 2014). The dispersed suspension was then wet-sieved through the corresponding sieves – i.e. depending on their aggregate class – until the rinsing water was clear. Organic material > 2 mm floating on the water was removed from LMa as it does not belong to POM (Six et al., 1998), and got discarded from the study since it was not present in all the samples (data not shown). The sand and POM mixture and the MaOM were oven-dried at 55 °C for 48 h and powdered. The OC and N contents were determined with a LECO CNS 2000 analyser (LECO Corporation, St. Joseph, MI, USA). In order to compare the OC and N concentrations of both POM and MaOM between aggregate-size classes, all values were expressed on a sand-free basis (g kg$^{-1}$ sand-free aggregate; Cambardella and Elliott (1992)). Mean mass recovery was 97.5 ± 2.6%.

### 2.6. SOC, TN concentrations and stable C isotopic composition in aggregate-size classes

Total N, OC concentrations and stable C isotope composition ($\delta^{13}$C) were determined on all aggregate-size classes using continuous-flow isotope ratio mass spectrometry in a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA) fitted with a zero-blank auto-sampler coupled via a ConFloIII to a ThermoFinnigan DeltaPlus-XL (Thermo Scientific, Waltham, MA, USA). Isotope compositions were expressed in δ notation, representing ‰ variation from the standard reference material:

$$\delta(‰) = \left[ \left( R_{sample} - R_{standard} \right) / R_{standard} \right] \times 1000 \tag{3}$$

where R is the ratio $^{13}$C/$^{12}$C. The standards used were Vienna Pee Dee Belemnite. The precision (standard deviation) of internal standards for elemental C and its stable isotopic composition were better than 0.06% and 0.3‰ respectively.

### 2.7. Enzyme activity analysis

The potential extracellular activities of the β-glucosidase and the NAGase enzymes were analysed. These are involved in the microbial degradation of cellulose and chitin, respectively.

The activity of the enzyme β-glucosidase was measured according to the procedure proposed by Hoffmann and Dedeken (1964), subsequently modified by Strobl and Traunmüller (1996). Briefly, 5 g of sieved (2 mm) bulk soil at 60% field capacity was incubated at 37 °C with 5 ml of β-glucosido-saligenin used as substrate and 10 ml of 2 *M* acetate buffer (pH 6.2) for three hours. The samples were then filtered through a Whatman filter paper (no. 42) and their absorbance at 578 nm was measured in a spectrophotometer (Shimadzu UV-1203, Shimadzu, Kyoto, Japan). β-glucosidase activity was expressed as μg saligenin g$^{-1}$ dw soil (3 h)$^{-1}$.

The activity of the enzyme NAGase was measured using the method proposed by Parham and Deng (2000). Briefly, 1 g of sieved (2 mm) bulk soil at 60% of field capacity was incubated at 37 °C with 1 ml of 10 mM of *ρ*-nitrophenyl-*N*-acetyl-β-*D*-glucosaminide solution and 4 ml of 0.1 *M* acetate buffer for one hour. After incubation, 1 ml of 0.5 *M* $CaCl_2$ and 4 ml of 0.5 *M* NaOH was added to halt the reaction, and the samples were mixed and filtered through a Whatman filter paper (no. 42). NAGase activity was determined as the colour intensity of the filtrate, measured with a spectrophotometer (Shimadzu UV-1203) at 405 nm and expressed as μg saligenin g$^{-1}$ dw soil (h)$^{-1}$.

### 2.8. Phospholipid fatty acid analysis

Phospholipid fatty acids (PLFAs) from sieved (2 mm) bulk soil samples were determined using a modified procedure by Frostegård et al. (1991) described in Koranda et al. (2011). Briefly, fresh soil samples were extracted with a mixture of methanol:chloroform:citrate buffer mixture (2:1:0.8, v:v:v). Neutral- and glycol lipids were removed using silica acid columns ISOLUTE ® SI Column (Biotage AB, Uppsala, Sweden) and PLFA were diluted in methanol. The extract was then evaporated to dryness and the PLFA were subjected to mild alkaline methanolysis. Dried fatty acid methyl esters were re-dissolved in isooctane, and concentrations were determined by a 6890 N gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a split/splitless inlet on a HP-5MS capillary column (50 m × 0.20 mm × 0.33 μm) and detected with a flame ionisation detector. A mixture of bacterial acid methyl esters (BAME mix, Supelco, Vienna, Austria) was used as a qualitative standard, and concentrations of single fatty acid methyl esters were calculated using the internal standard (19:0) peak as a reference (19:0, Sigma-Aldrich, USA). The different PLFAs were classified by microbial groups (Table 2) and expressed as concentration (nmol g$^{-1}$ dw soil).

### 2.9. Data analyses

Stocks of soil OC and N (Mg ha$^{-1}$) were calculated using the following equation:

$$Stock_{[OC,N]} = BD \times D \times FE \times [OC, N]/10 \tag{4}$$

where *BD* is the soil bulk density (g cm$^{-3}$), *D* is the thickness of the soil sample (cm), *FE* is the fine-earth mass per soil sample (g g$^{-1}$) and *[OC, N]* are the contents of OC or N in the fine earth (mg g$^{-1}$) respectively. The OC and N stocks in each aggregate class were calculated as:

**Table 2**
Functional groups of different PLFAs found in the soil samples.

| Functional group | PLFA group | Specific PLFA markers | References |
|---|---|---|---|
| *Fungal groups* | | | |
| Saprophytic fungi | Polyunsaturated PLFAs | 18:3ω6[a],<br>18:2ω9,12[b, c] | Potthoff et al. (2006) [a]<br>Ratledge and Wilkinson (1988) [b]<br>Yu et al. (2009) [c] |
| Arbuscular mycorrhizal fungi (AMF) | | 16:1ω5[c] | Olsson (1999)<br>Olsson et al. (1995) [c] |
| *Bacterial groups* | | | |
| Gram-positive (Gram+) bacteria | saturated PLFAs | i14:0[d], i15:0[d], a15:0[d], i16:0[d], a16:0[d], i17:0[d], a17:0[d], 17:0 10Me[d] | Bach et al. (2010) [d] |
| Gram-negative (Gram −) bacteria | cyclopropyl and monounsaturated PLFAs | 16:1ω9[f], 16:1ω11[d], 17:1ω9[f], 18:1ω11[f], cy18:0[g], cy19:0ω9[h], cy17:0 (9/10)[i] | Bach et al. (2010) [d]<br>Schindlbacher et al. (2011) [f]<br>Koranda et al. (2013) [g]<br>Dennis et al. (2013) [h]<br>Wild et al. (2014) [i] |
| F/B ratio | Multiple groups | Fungal to bacterial groups | |
| Actinomycetes | 10Me-PLFAs | 16:0 10Me[d], 18:0 10Me[d] | Bach et al. (2010) [d] |
| Protozoan | | 20:0 | Schindlbacher et al. (2011) [f] |

$$Stock_{[OC,N]i} = BD \times D \times FE \times A_i \times [OC, N]_i / 10 \tag{5}$$

where $A_i$ is the content of aggregate *i* in the soil matrix and *[OC, N]$_i$* are the OC or N contents of the *i* fraction.

All statistical analyses and graphic representations were done using the R software (version 3.2.3, R Core Team (2015)). All data were tested for differences between vegetation cover types (SL, SL-FO, FO) with generalized linear mixed models (lme4 package, Bates et al. (2015)) with gamma distribution error. For data on bulk soil (SOC, total N, C:N ratios, PLFA and enzymatic activities), vegetation cover was used as the fixed factor and plot was introduced as a random factor to include within-site correlations in the model. Analyses were also performed within aggregate-size classes, with aggregate-size class being introduced as the fixed factor in the model. The Tukey post-hoc multiple comparison test was done (multcomp package, Hothorn et al. (2016)) to obtain the differences between vegetation cover types after generalized linear mixed models. Normality and homogeneity of the variance of the residuals were analysed with the Shapiro-Wilk (stats package, R Core Team (2015)) and Levene test (car package, Fox and Weisberg (2011)) respectively. The non-parametric Kruskal-Wallis test (stats package, R Core Team (2015)) was used to assess differences in soil $\delta^{13}$C between land covers due to the lack of assumptions on the normality and homogeneity of variances. The significance level was set at $p < 0.05$ for all tests, unless noted otherwise in the text.

Redundancy analysis (RDA, vegan package, Oksanen et al. (2017)) was performed on the data for the concentration of microbial groups, enzyme activity and aggregate properties to determine the relationships between soil microbial community and environmental conditions. A preliminary detrended correspondence analysis was performed to check that the gradient length of the first axis was lower than 4 standard deviation units; if this was the case, linear responses can be expected and RDA is suitable for use (Lepš and Šmilauer, 2003). Due to the difference in both their magnitude and units, microbial and environmental data were centred and scaled to unit variance. Forward selection procedure, based on a Monte Carlo permutation with 1000 iterations, was applied to determine the most significant discriminating variables in the composition of individual PLFAs for use in the final analyses. All results are expressed as mean ± 1 standard deviation.

## 3. Results

### 3.1. Soil OC and N concentrations and stocks

The concentrations of SOC ranged from 35.8 to 40.4 mg g$^{-1}$ (Table 3) with no significant differences shown between land cover types. Total N concentrations ranged from 2.1 to 3.1 mg g$^{-1}$ with greater values observed in SL than in FO (Table 3). The C:N ratio showed a decreasing trend from FO to SL (Table 3).

SOC stocks did not differ significantly between the three land covers, with values ranging from 8.4 to 12.4 Mg C ha$^{-1}$ (Table 3). Significantly higher soil N stock was observed under SL (0.9 Mg N ha$^{-1}$) compared to SL-FO (0.6 Mg N ha$^{-1}$) and FO (0.5 Mg N ha$^{-1}$).

### 3.2. Aggregate-size class distribution

Small macroaggregates (SMa) accounted for >50% of the soil mass while Mi, LMa and silt + clay fractions represented 20–25%, 10–15% and 5–10% of the soil mass respectively (Fig. 1). Soils in FO and SL-FO land covers presented significantly larger amounts of SMa than those of SL. FO soils showed a higher LMa mass, but lower Mi mass than SL-FO or SL soils (Fig. 1).

FO soils showed significantly higher MWD (1.34 ± 0.11 mm) than soils under other land covers (1.20 ± 0.07 and 1.15 ± 0.10 mm for SL and SL-FO soils respectively). FO soils also showed significantly greater MGD (0.74 ± 0.08 mm) than SL (0.61 ± 0.06 mm) and SL-FO soils (0.61 ± 0.05 mm).

### 3.3. Aggregate-associated organic carbon, nitrogen, C:N ratios and $\delta^{13}$C values

Regardless of land cover, OC and N concentrations followed a common discernible pattern with aggregate size, according to s + c > Mi > SMa > LMa (Fig. 2a, 2b). FO soils showed several-fold higher OC concentration in LMa than SL and SL-FO soils. However, FO and SL-FO soils had a lower OC concentration associated to s + c class than SL soils (Fig. 2a). Both N concentrations associated to Mi and s + c classes in FO soils were significantly lower than in SL soils (Fig. 2b). The C:N ratios

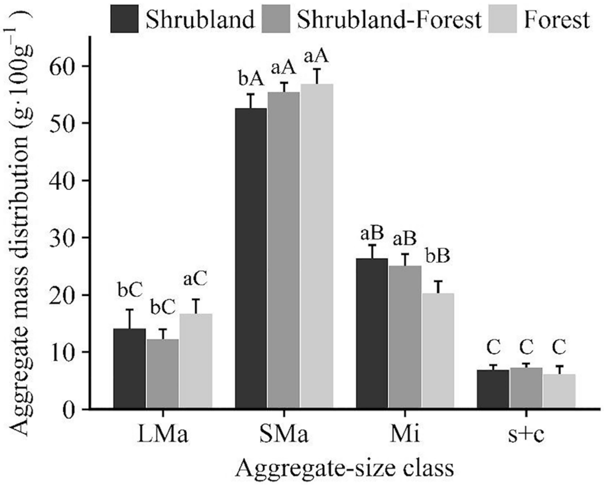

**Fig. 1.** Mass distribution (g 100 g$^{-1}$ soil) among the different aggregate-size classes. Lowercase letters denote significant differences ($p < 0.05$) between land covers in each aggregate-size class, and capital letters show significant differences ($p < 0.05$) between aggregate-size classes in each land cover. Bars represent mean values ± standard deviation. LMa, large macroaggregates (6–2 mm); SMa, small macroaggregates (2–0.250 mm); Mi, microaggregates (0.250–0.053 mm); s + c, silt + clay size particles (<0.053 mm).

**Table 3**
Soil organic carbon and total nitrogen concentrations, C:N ratios, and stocks for each land cover.

| Land cover | SOC (g kg$^{-1}$) | Total N (g kg$^{-1}$) | C:N ratio | SOC stock (Mg ha$^{-1}$) | Total N stock (Mg ha$^{-1}$) |
|---|---|---|---|---|---|
| SL | 40.4 ± 9.9 | 3.1 ± 0.6a | 13.1 ± 0.6c | 12.4 ± 3.1 | 0.9 ± 0.2a |
| SL-FO | 36.0 ± 4.5 | 2.5 ± 0.3ab | 14.3 ± 0.8b | 8.4 ± 1.5 | 0.6 ± 0.1b |
| FO | 35.8 ± 7.3 | 2.1 ± 0.4b | 17.2 ± 1.4a | 8.8 ± 3.3 | 0.5 ± 0.2b |

Values are means ± standard deviation. Letters denote significant differences ($p < 0.05$) between land covers. SL, Grassland/Shrubland; SL-FO, Shrubland-Forest; FO, Forest.

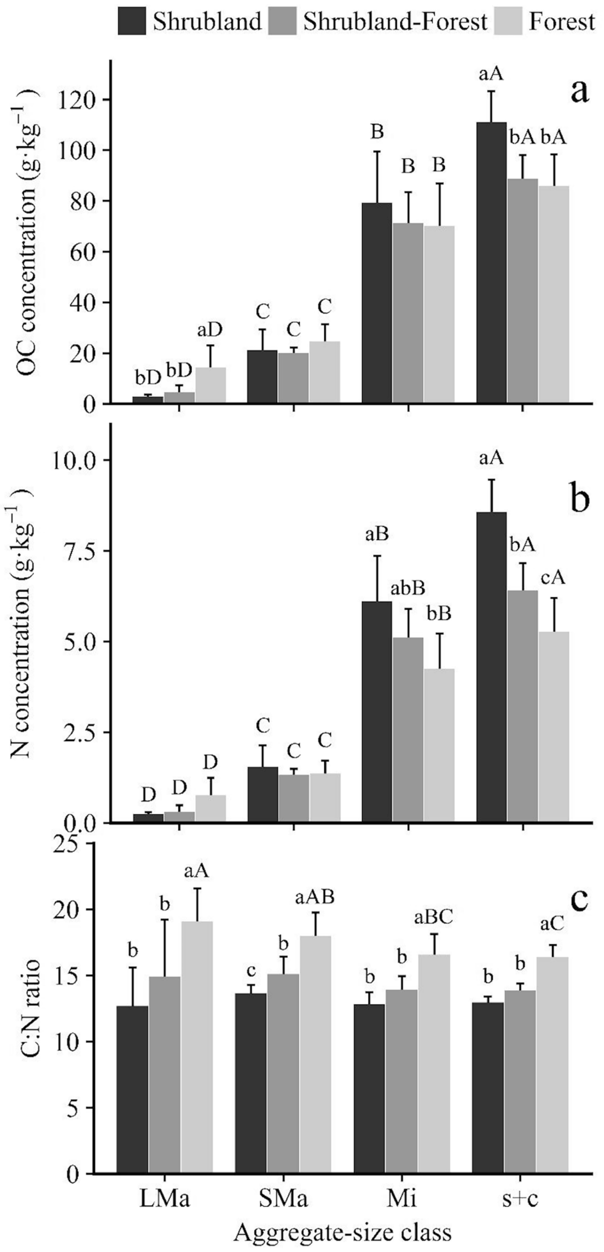

**Fig. 2.** Concentrations (g $kg^{-1}$) of a) associated organic carbon (OC), b) associated nitrogen (N), and c) the C:N ratio between the aggregate-size classes in the ecotone. Lowercase letters denote significant differences ($p < 0.05$) between land covers in each aggregate-size class, and capital letters show significant differences ($p < 0.05$) between aggregate classes in each land cover. Bars represent mean values ± standard deviation. Aggregate-size acronyms are as in Fig. 1.

were highest in FO soils for all aggregate classes. FO soils showed a decreasing trend in C:N ratios with decreasing aggregate-size class (Fig. 2c).

Both POM-C and POM-N concentrations in SMa were significantly higher than in LMa and Mi in all land cover types. Concentrations of C and N in POM showed no significant differences across land cover types (Fig. 3a, c). Unlike POM, both the MaOM-C and MaOM-N significantly decreased with increasing aggregate-size class (Fig. 3b). Organic C and TN concentrations in MaOM (MaOM-C and MaOM-N respectively) showed significant differences in LMa between land cover types (FO > SL Fig. 3b and d). In the Mi class, the MaOM-N showed a higher concentration under SL and SL-FO than under FO soils (Fig. 3d). In all the aggregate-size classes, the C:N ratios in POM and MaOM were greater in soils under FO than in those under SL or SL-FO (Fig. 3e and f), with the exception of POM-C:N in LMa, whose difference was not significant.

The OC and N stocks associated to LMa were significantly greater in soils under FO than in those under SL or SL-FO, while OC and N stocks associated to Mi and s + c were significantly greater in soils under SL than under SL-FO or FO land covers (Fig. 4a and b). In terms of aggregate-size class, both OC and N stocks were greater in Mi than in LMa, whereas SMa and s + c had intermediate values.

$\delta^{13}C$ values in the various aggregate-size classes ranged from −25.9 ± 0.2 to −24.9 ± 0.1 ‰ (Table 4). The smallest aggregate-size classes (Mi and s + c) presented significantly higher $\delta^{13}C$ values in SL cover compared to FO stands, which consistently showed the lowest $\delta^{13}C$ values of all the vegetation types considered (Table 4).

### 3.4. *Soil microbial community structure and enzymatic activities*

Total soil microbial PLFA content under SL was significantly higher than under SL-FO and FO covers (Table 5); this pattern was largely explained by differences in Gram + bacteria, which were significantly more abundant in SL soils. No significant differences were observed in Gram– bacteria, AMF, saprophytic fungi and actonmycetes contents between the different land covers. The highest abundance of protozoa occurred in FO soils (Table 5). Enzymatic activity analyses showed that β-glucosidase rates were significantly higher in SL than in FO soils, while no differences were found in NAGase activity between land covers (Table 5).

RDA explained over 60% of the variance in the relationship between aggregate properties, β-glucosidase activity and the concentration of microbial groups in the land covers studied (Fig. 5). The first two constrained axes were highly significant ($p < 0.001$) and accounted for over 55% of the total variance (34.8% and 21.7% for axis 1 and 2 respectively). Gram + bacteria were positively related to MaOM-N concentration in both SMa and Mi aggregates, OC in the s + c aggregate-size class and to β-glucosidase activity, but clearly opposed to the POM-C and MaOM-C concentration in the LMa aggregates (Fig. 5a).

Forest soils were associated with a higher abundance of protozoa, and greater MWD and C concentration in MaOM aggregates (Fig. 5b). Shrubland soils were associated with greater OC concentrations in bulk and the s + c aggregate-size class. This vegetation cover also showed a strong relationship with MaOM-N concentrations in both SMa (2–0.25 mm) and Mi (250–53 μm) size classes, and with greater β-glucosidase activity (Fig. 5b).

## 4. Discussion

### 4.1. *Influence of vegetation and microclimatic conditions on soil structure*

Our study indicates a strong influence of vegetation on soil aggregation dynamics. As hypothesised, forest soils showed a comparatively higher abundance of large aggregate-size fractions and greater MWD and MGD values, which were most likely caused by the increase in large root biomass, exudates, and litter inputs that favour the aggregation of soil particles (Tisdall and Oades, 1982; Bronick and Lal, 2005). These findings are consistent with those of Guidi et al. (2014b) who also found a positive relationship between forest cover and aggregate-size along a grassland-forest transition in the southern Alps. Also of particular note is the negative effect that tree expansion had on micro-aggregate stability (Fig. 1), which will be further discussed in the context of SOM dynamics and microbial community structure in subsequent sections.

In addition to the influence of vegetation type on soil structure, we observed that aggregate-associated OC and N contents increased with decreasing aggregate size in all land covers (Fig. 2). This was contrary to both our initial expectations and the results reported by Guidi et al (2014b). The most plausible explanation for this large discrepancy is the coarse nature of the soils investigated in the present study (i.e. sand

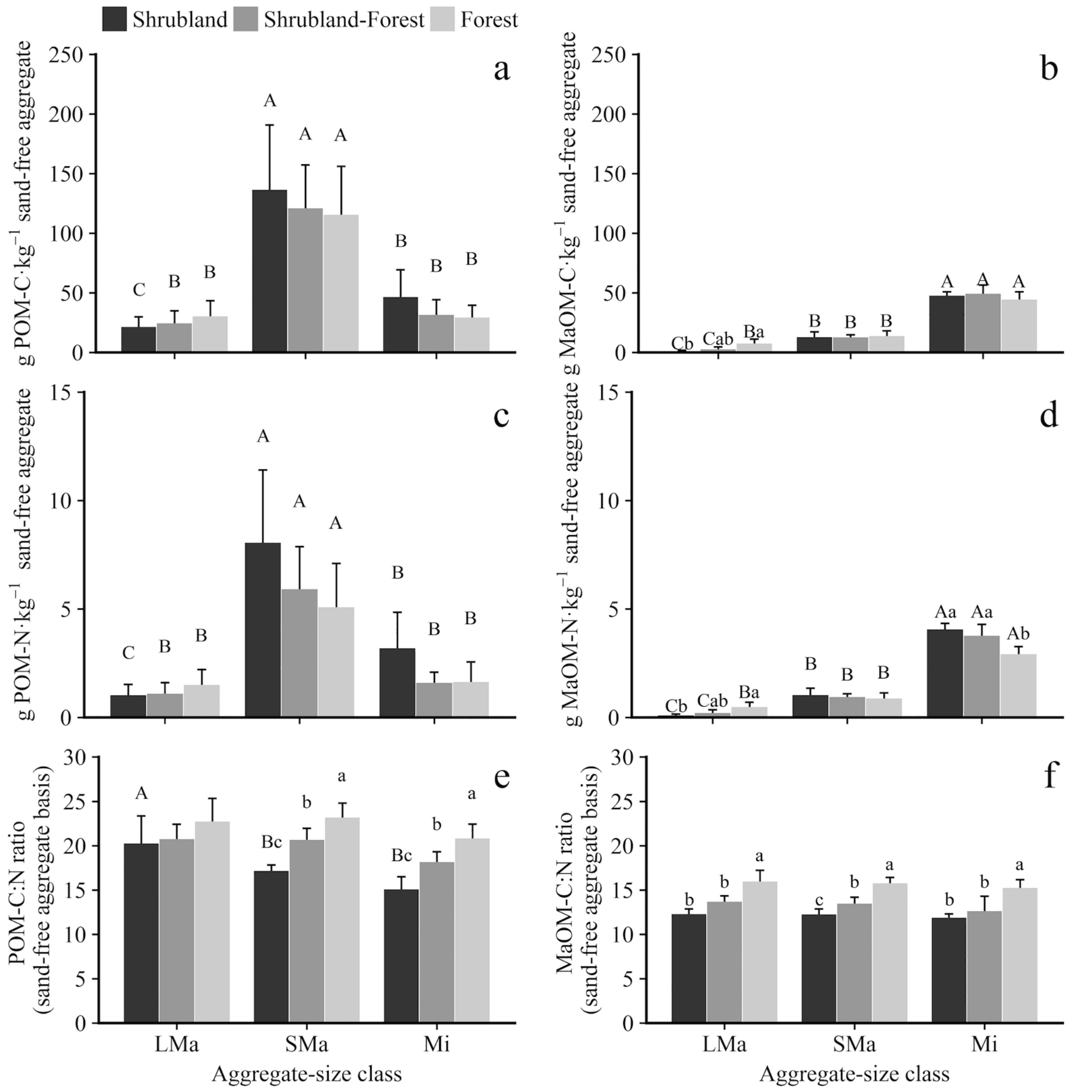

**Fig. 3.** Concentrations (g kg$^{-1}$ sand-free aggregate) of a) particulate organic carbon (POM-C), b) mineral-associated organic carbon (MaOM-C), c) particulate organic nitrogen (POM-N), and d) mineral-associated organic nitrogen (MaOM-N), and carbon to nitrogen ratios in e) particulate organic matter (POM-C:N) and f) mineral-associated organic matter (MaOM-C:N) across the different aggregate-size classes in the ecotone. Mean values ± standard deviations are shown. Lowercase letters denote significant differences ($p < 0.05$) between land covers in each aggregate-size class, and capital letters show significant differences ($p < 0.05$) between aggregate-size classes in each land-cover type. Aggregate-size acronyms are as in Fig. 1.

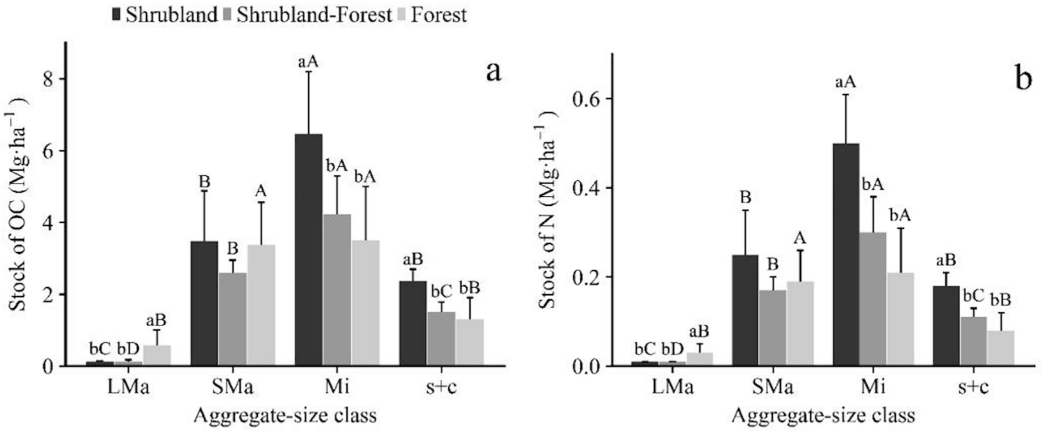

**Fig. 4.** Topsoil stocks (Mg ha$^{-1}$) of a) organic carbon (OC) and b) nitrogen (N) in the different aggregate-size classes in the ecotone. Bars represent mean values ± standard deviation. Lowercase letters denote significant differences ($p < 0.05$) between land covers in each aggregate-size class, and capital letters show significant differences ($p < 0.05$) between aggregate-size classes in each land-cover type. Aggregate-size acronyms are as in Fig. 1.

**Table 4**

$\delta^{13}$C contents (‰) in the aggregate-size classes along the different land covers in the ecotone.

| Land cover | Aggregate-size class | | | | | |
|---|---|---|---|---|---|---|
| | LMa | SMa | Mi | | s + c | |
| SL | n.d. | −25.1 ± 0.2 | −24.9 ± 0.0 | a | −24.9 ± 0.1 | a |
| SL-FO | n.d. | −25.5 ± 0.4 | −25.2 ± 0.1 | ab | −25.3 ± 0.1 | ab |
| FO | −25.6 ± 0.3 | −25.7 ± 0.4 | −25.9 ± 0.2 | b | −25.8 ± 0.2 | b |

Values are means ± standard deviation. Letters denote significant differences ($p < 0.05$) between land covers. SL, Grassland/Shrubland; SL-FO, Shrubland-Forest; FO, Forest; LMa, large macroaggregates; SMa, small macroaggregates; Mi, microaggregates; s + c, silt and clay size particles. n.d., not determined due to the absence of a reliable value.

contents > 69%; Table 1). Indeed, compared to sandy substrates, fine-textured soils have a higher capacity to promote physical aggregation, which confers OM an enhanced physical protection against microbial decomposition. This is because smaller particles have a larger surface area that promotes the stabilization of OM (von Lützow et al., 2006; Saiz et al., 2012). Small macroaggregates were the most abundant aggregate-size class in all vegetation types and showed the highest concentration of POM-C (Figs. 1; 3a). This observation further stresses the importance of POM in the formation of these structures, which confer this relatively fresh OM physical protection against microbial attack and mineralization (Six et al., 1998; Pikul et al., 2007; Meyer et al., 2012; Wu et al., 2012).

In addition to direct biotic drivers, afforestation and woody encroachment in cold mountainous regions generate a more favourable microclimate for the activity of microorganisms as a result of the insulating effect provided by the forest canopy and the warmer soils favoured by deeper snow layers during winter (Hagedorn et al., 2014). Indeed, winter snow may reduce the episodes of soil freezing and subsequent thaw cycles that indirectly affect C and N dynamics by altering soil aggregate stability, which is a key factor controlling soil sustainability (Kværnø and Øygarden, 2006) and SOM cycling (Hagedorn et al., 2010). Additionally, Mediterranean alpine ecosystems may also suffer from water deficits during the commonly dry summer months (Sánchez Palomares et al., 1999), which may be particularly relevant in those ecosystems established in coarse-textured soils. In this context, the presence of trees may have also had a positive effect on maintaining sustained soil water conditions suitable for the activities of SOM decomposers, as their thick forest floor and dense canopies reduce topsoil water evaporation through shading. Indeed, research work conducted on coarse-textured soils in semi-arid regions has shown that tree canopy-related effects enhance SOM decomposition processes (Saiz et al., 2015; Saiz et al., 2016).

Vegetation shifts in this Mediterranean alpine ecosystem exerted a significant impact on soil aggregation dynamics, which were likely caused by vegetation-related variations both in SOM inputs and soil microclimatic conditions.

### 4.2. Variation of soil $\delta^{13}$C values and C:N ratios along the ecotone

Table 4 shows aggregate–size fractions in grassland/shrubland soils having higher $\delta^{13}$C values than their forests counterparts, which may suggest that carbohydrates in these soils are more heavily metabolized than those of forest stands. However, there are isotopic fractionation effects that preclude a straightforward interpretation of these data. Potential reasons include factors associated with microbial re-processing, and vegetation-related factors including light intensity, humidity, and the re-utilization during photosynthesis of previously respired low $^{13}$C-$CO_2$ within the forest canopy (Bird et al., 1994; Saiz et al., 2015; Gerschlauer et al., 2019). Therefore, these factors may have also had an impact on the lower $\delta^{13}$C values observed in stands with trees (forests and shrubland-forests).

Coniferous litter typically has a wide C:N ratio and a complex chemical structure (Berg, 2000; Pérez-Cruzado et al., 2014), which may have greatly contributed to the higher soil C:N ratios observed in forest stands across all aggregate-sizes (Figs. 2c; 3e-f) as it was also reported for bulk soils at the regional level (Ortiz et al., 2016). Considering the C:N ratios obtained for aggregate–size fractions in combination with the corresponding isotopic results support the notion that forest soils contain fresher, less decomposed OM than the other vegetation covers (Ortiz et al., 2016). Nevertheless, tree-dominated sites showed the lowest OM stocks in the smallest fractions (Fig. 4), which taken together with their significantly lower $\delta^{13}$C values and higher C:N values, further reflect the detrimental effect that vegetation shifts in this alpine ecosystem pose on soil microaggregate stability (Guidi et al., 2014b). It is worth noting that the comparatively high SOM dynamics (i.e. fast turnover rates) common of topsoils, make quite difficult to formulate unbiased claims of significant differences in $\delta^{13}$C values between aggregate sizes being attributed to differential preservation of OM, as it would normally be the case at deeper soil intervals, where a wide range of fresh and highly processed SOM substances coexist. Therefore, it is advisable to assess aggregate-size isotopic results in combination with other soil parameters.

### 4.3. Variations in microbial community structure and enzymatic activities

Our analyses show that PLFA differences between land covers were dominated by the large variations in the concentration of Gram + bacteria, which were more abundant in grassland/shrubland soils compared to those of forests (Table 5). These results are consistent with the general understanding of the various patterns of substrate preference exhibited by Gram + and Gram- bacteria, as well as with the different patterns in soil C:N ratios observed along the ecotone. Gram + bacteria tend to prefer old, well-processed SOM, whereas Gram- bacteria are thought to favour fresher plant inputs as a C source (Fierer et al., 2003; Potthast et al., 2012; Smith et al., 2014). Our results strongly support this microbial substrate preference, since RDA analyses showed that higher contents of Gram + bacteria were associated to more metabolized forms of C and N in the small aggregate-size fractions of grassland/shrubland soils (Fig. 5).

The β-glucosidase activity in soils is closely related to the quantity and quality of SOM, which may explain the progressively lower enzymatic activities observed along the vegetation sequence (Table 5). The high amounts of lignified litter biomass characteristic of tree vegetation and its comparatively high C:N ratios (Hiltbrunner et al., 2013) are likely to have resulted in the low β-glucosidase values observed in forest soils. Furthermore, forests also exhibited the lowest soil bacterial population among all land covers in the ecotone. These latter facts may well contribute to low microbial substrate use efficiency (Cotrufo et al., 2013) in tree-dominated stands, which has been suggested to be a factor for the reduced soil aggregate stability observed following forest expansion on former grasslands (Guidi et al., 2014b). This agrees well with our study which shows that increased tree cover had a significantly negative impact on micro-aggregate stability (Fig. 1). Conversely, aggregate stabilization is greatly promoted by the presence of fine roots, and it is well known that compared to trees, grasses produce higher amounts of fine roots with comparatively greater lengths and faster turnover rates (Guo et al., 2007). Also, the higher β-glucosidase activity, and the greater presence of Gram+ bacteria observed in grassland/

**Table 5**
Concentrations of total PLFAs (nmol $g^{-1}$soil dw), gram-positive bacteria (Gram +), gram-negative bacteria (Gram -), arbuscular mycorrhizal fungi (AMF), saprophytic fungi (Sap. fungi), actinomycetes, protozoa, fungal to bacterial ratio (F:B) and enzyme activities of β-glucosidase (μg saligenin $g^{-1}$ dw soil 3 $h^{-1}$) and *N*-acetyl-β-glucosaminidase (NAGase) activity (μg saligenin $g^{-1}$ dw soil $h^{-1}$).

| Land cover | Total PLFA | Total bacteria | Gram+ | Gram- | AMF | Sap. fungi | Actinomyc. | Protozoa | F:B | β-glucosidase | NAGase |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SL | 389.3 ± $69.1^{a}$ | 269.2 ± $47.3^{a}$ | 220.0 ± $44.8^{a}$ | 50.4 ± 13.1 | 2.5 ± 0.9 | 9.6 ± 2.1 | 17.9 ± 7.0 | 3.1 ± $1.1^{b}$ | 0.04 ± $0.01^{b}$ | 298.2 ± $30.1^{a}$ | 552.5 ± 212.9 |
| SL-FO | 303.0 ± $35.3^{b}$ | 180.4 ± $15.6^{ab}$ | 133.8 ± $16.1^{b}$ | 46.7 ± 10.1 | 2.2 ± 0.7 | 9.0 ± 2.6 | 14.9 ± 3.3 | 4.0 ± $0.5^{b}$ | 0.05 ± $0.02^{ab}$ | 258.0 ± $35.9^{ab}$ | 587.7 ± 182.3 |
| FO | 283.3 ± $57.5^{b}$ | 140.8 ± $30.5^{b}$ | 80.7 ± $22.0^{c}$ | 60.2 ± 13.4 | 2.6 ± 0.9 | 9.7 ± 3.4 | 16.0 ± 3.7 | 7.2 ± $1.3^{a}$ | 0.07 ± $0.02^{a}$ | 222.0 ± $41.2^{b}$ | 613.8 ± 190.6 |

Values are means ± standard deviation. Letters denote significant differences ($p < 0.05$) between land covers.

shrubland soils, demonstrates that soil microorganisms under this vegetation cover have a higher capacity to metabolize carbohydrates, which suggests that this soil may be comparably more depleted in easily decomposable substrates. On the other hand, the similar NAGase activities observed between the various vegetation types (Table 5) was likely due to the lack of significant differences in some of the key enzymatic drivers pointed out by Sinsabaugh et al. (2008), which include soil pH, SOC contents and fungal biomass (Table 5).

While vegetation shifts did not result in significant differences in topsoil OC contents between land covers, they induced changes in soil enzymatic activities and the structure of the soil microbial community, which had a significant impact in soil aggregation dynamics. Our results strongly suggests that tree establishment in this Mediterranean alpine ecosystem negatively impacts microaggregate stability.

### 4.4. *Forest vegetation promotes the formation of macroaggregates*

Compared to neighbouring areas dominated by grasslands or shrublands, the higher amounts of litterfall (Archer et al., 2001) and larger root diameters (Jastrow et al., 1998) usually observed under forest vegetation are described as the main causes for most of the OC in forest soils being occluded within macroaggregates (Caravaca et al., 2004). POM-C, which is generally considered a labile C form (Cambardella and Elliott, 1992; Gregorich et al., 2006) did not vary between land covers, although the RDA highlighted a relationship between forest stands and POM-C concentration in large macroaggregates, indicating that soils under forest vegetation contain comparatively fresher, less decomposed OM inputs. We suggest that the comparatively larger abundance of macroaggregates in forest soils (Fig. 2a) may contribute to a higher degree of physical protection against microbial attack (Six et al., 2000), therefore explaining the higher presence of less decomposed OM at these sites.

We report that the increasing presence of trees favoured the predominance of fungal groups over bacterial communities in the soils studied (Table 5). Indeed, fungal biomass, its residues and secondary metabolites have been shown to have lower decomposition rates than those of bacteria (Bailey et al., 2002; Six et al., 2006; Smith et al., 2014). Fungi also play an important role in soil aggregation dynamics since the extracellular polysaccharides produced by their mycelium act as cement between soil particles (Oades and Waters, 1991). Aggregate-size classes and their associated pores greatly control the links between organisms in a soil food web (Elliott and Coleman, 1988). A number of studies have reported decreased fungal abundance and fungal to bacterial (F:B) ratio with decreasing particle size (Chiu et al., 2006; Briar et al., 2011). This trend agrees well with the established perception that fungi are more prominent in macroaggregates while bacteria predominate in microaggregates, since fungal hyphae are unable to penetrate the small pores of the latter (De Gryze et al., 2005). Furthermore, the higher soil C:N ratios observed in forest stands correspond well with observations by Strickland and Rousk (2010) who reported that fungal C:N ratios are higher than those of bacterial communities. It is therefore to be expected that an increase in F:B ratios with tree encroachment may lead to greater soil macro-aggregation (MacDonald et al., 2009; Smith et al., 2014). Moreover, Garcia-Franco et al. (2015) have shown that, following the afforestation of shrublands, the increase in SOC pools linked to changes in microbial activity and fungal community structure promote the formation of macroaggregates. It is quite likely that increased macroaggregation in forest stands was a major contributor to the SOC accumulation observed in the full A-horizon (~25 cm) in this Mediterranean alpine ecosystem after the long-term conversion from grassland/shrubland to forest (Ortiz et al., 2016).

Finally, we feel that it is appropriate to integrate our work within the framework of the latest relevant advances in the literature. While the significance of aggregate formation in SOM preservation has been acknowledged for decades (Oades, 1984; Six et al., 2004), recent research progress argues about the superior significance of mineral-

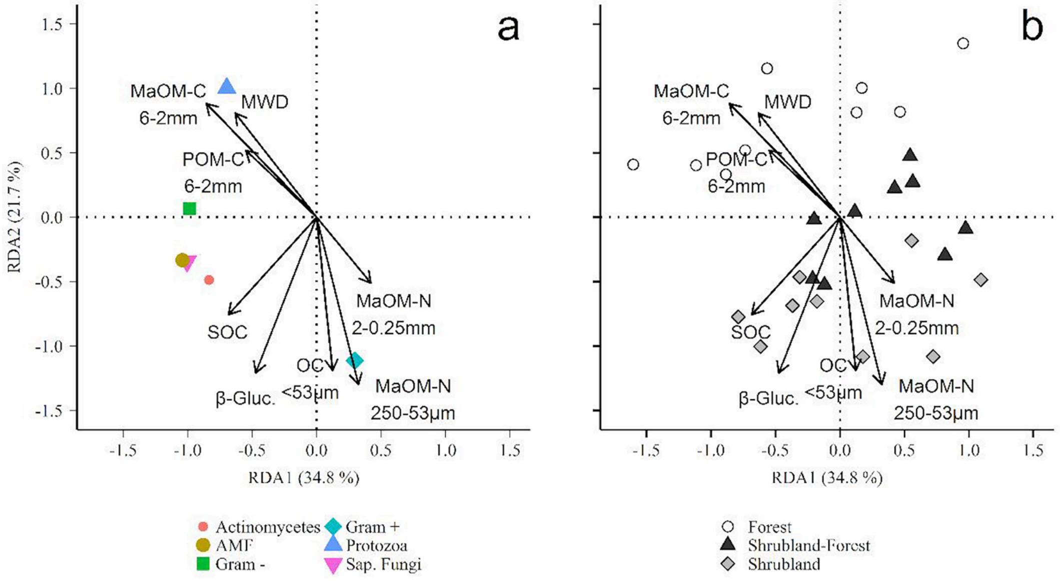

**Fig. 5.** Redundancy analysis (RDA) ordination biplot showing the relationship between aggregate-size classes, β-glucosidase activity, and: a) concentration of soil microbial functional groups; b) land cover types. Sap. Fungi, saprophytic fungi; Gram +, Gram-positive bacteria; Gram -, Gram-negative bacteria; AMF, arbuscular mycorrhizal fungi; SOC, soil organic carbon; SOC, soil organic carbon; LMa, large macroaggregates (6–2 mm); SMa, small macroaggregates (2–0.250 mm); Mi, microaggregates (0.250–0.053 mm); s + c, silt + clay size particles (<0.053 mm); MWD, mean weight diameter; POM-C, particulate organic carbon; MaOM-C, mineral-associated organic carbon; MaOM-N, mineral-associated organic nitrogen.

associated OM on SOC dynamics (Cotrufo et al., 2019). Within this context, Fig. 4 shows that SOC and TN are mainly distributed in microaggregates and small aggregate-size classes. Such distribution may likely be attained through different mechanisms, whereby micro-aggregates may be more dominated by mineral-associated OM (as suggested by the higher OC contents in s + c and micro-aggregate MaOM; Fig. 2a, 3b), while small macro-aggregates may be more influenced by POM (higher OC contents in small macro-aggregate POM, Fig. 3a). Even if our data suggest that this may be a plausible argument, the exact mechanisms involved need to be explored in greater detail.

## 5. Conclusions

Vegetation shifts led to a different physical stabilization of topsoil OM within aggregates in this Mediterranean alpine ecotone. Compared to the grassland/shrubland sites, the afforestation and subsequent tree encroachment in neighbouring areas resulted in an increase in soil macroaggregate contents and greater mean weight diameters. Although no differences were found in SOC stocks in bulk samples, the establishment and subsequent expansion of trees had a detrimental effect on the stability of microaggregates, negatively impacting the OM contents of these small aggregate-sizes. Our work independently confirms the observations by Guidi et al. (2014b) about the stability of small aggregates being negatively affected by expansion of woody vegetation on grassland alpine ecosystems. Small macroaggregates appear to play a key role in aggregate dynamics in all the studied soils, as they contained significantly higher concentrations of POM (C and N). However, POM represents the most labile pool of OM, and as such, it is the most exposed to potential mineralisation.

The observed changes in soil aggregation and microbial community structure caused by vegetation shifts strongly suggest that SOM may become more susceptible to environmental change. This fact could be particularly relevant in these alpine regions, as the projected increase in global temperatures may result in potentially large SOM losses due to the strong temperature sensitivity to decomposition that is usually reported in low-temperature (i.e. high-elevation) ecosystems (Schindlbacher et al., 2010; Schindlbacher et al., 2011; Gutiérrez-Girón et al., 2015; Blagodatskaya et al., 2016; Gerschlauer et al., 2019). This study shows that the afforestation and potential vegetation shifts experienced in Mediterranean alpine grasslands leads to distinct changes in soil microbial communities, aggregation and SOM dynamics. These trends may get exacerbated in the current context of global change, which grants further research in the impact caused by extreme environmental events in these relatively understudied ecosystems.

## Declaration of Competing Interest

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

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