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Corresponding Author: Dr. Sara Marinari, PhD

Corresponding Author's Institution: University of Tuscia

First Author: Ruxandra Papp

Order of Authors: Ruxandra Papp; Sara Marinari, PhD; Maria Cristina Moscatelli; Enio Campiglia; Roberto Mancinelli; Emanuele Radicetti; Marcel G.A. van der Heijden; Raphael Wittwe; Bruce Pearce; Nick Fradgeley; Goran Bergkvist; Maria Renate Finckh

Abstract: The goals of conservation agriculture are to preserve and enhance the soil resource base and the environment. Subsidiary crops (SCs), such as Trifolium, Medicago, Vicia, Brassica, Raphanus spp, are important components of conservation agriculture since they maintain the soil resource. However, the importance of SC species and environment on soil microbial communities are not well known. The overall objective of this study was to assess the effect of various subsidiary crops cultivation on soil microbial biomass and activity at four sites across Europe. The experiments were conducted during 2014 and 2015 at sites in the Nemoral (Sweden SLU), Oceanic (United Kingdom ORC), Continental (Switzerland AGS) and Mediterranean north (Italy UNI) pedo-climatic zones. The specific objectives were to determine: (i) the effect of SC growth on soil microbial biomass and activity, ii) the site-specific effect of SC growth on soil biochemical properties. The SCs consisted of leguminous or brassicaceous species sown after wheat harvest, or clover species under-sown in wheat. At 0-30 cm depth, microbial carbon and nitrogen increased under SCs at most sites indicating that SCs cultivation may favor soil biological fertility. Effects of SCs were similar in the pedo-climatic zones where air temperatures are never below 0 °C (ORC and UNI). Arylsulphatase was the most sensitive enzyme to legumes in the Mediterranean north (UNI). Chitinase activity was enhanced by SCs in the Oceanic and Nemoral pedo-climatic zones. High precipitation and the low average temperature, typical of Continental and Nemoral zones, may represent limiting factors for soil enzyme activity under all selected SCs. Among the four pedo-climatic zones, the Mediterranean north represented the most suitable environment to promote SC growth and soil coverage. This study showed that SC cultivation affects soil quality enhancing biochemical activity; however the SCs effect were influenced by the different pedo-climatic conditions.

Viterbo February 26th , 2018

To the Editor of
Soil Tillage Research

Dear Editor,

We wish to submit the article entitled "**Short-term changes in soil biochemical properties as affected by subsidiary crop cultivation in four European pedo-climatic zones"** revised according to the reviewers comments.

We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

We declare that we do not have conflicts of interest to disclose.

Please address all correspondence concerning this manuscript to me.

We thank the anonymous referees for the precious comments that improved the manuscript.

Sincerely,

Dr. Sara Marinari
DIBAF – University of Tuscia
Via S. Camillo de Lellis

Viterbo 01100 - ITALY

marinari@unitus.it

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Reviewers' comments:

Reviewer #1:
Authors have revised the paper based on the comments of reviewers. My suggestion is that the manuscript now meets the requirements for Soil & Tillage Research.
One comment:

Materials and Methods
-Page 4: numbering of equation is missing

Done

Reviewer #4:

L414, Delete the comma after "zones".
Done
L422, effects;
Done
Table 3, Provide the acronyms of soil properties. Delete "For soil properties acronyms see table 3".
Done

Highlights

1. Subsidiary crops cultivation affects soil quality enhancing biochemical activity.
2. Subsidiary crops short-term effect on soil were similar in the mild pedo-climatic zones.
3. High rainfall and low temperature may reduce the effect of subsidiary crops growth on soil.
4. The Mediterranean north was the most suitable climate to promote leguminous growth.
5. Soil arylsulphatase and chitinase activities were sensitive to subsidiary crops cultivation.

Short-term changes in soil biochemical properties as affected by subsidiary crop cultivation in four European pedo-climatic zones

Papp R.[a], Marinari S. [a*] Moscatelli M.C. [a]

[a] Department for Innovation in Biological, Agro-food and Forest system, University of Tuscia, Via S. Camillo de Lellis snc, 01100 Viterbo, Italy

van der Heijden M.G.A. [b], Wittwer R. [b]

[b] Agroscope, Research division Agroecology and Environment, Plant-Soil-Interactions, Reckenholzstrasse 191, 8046 Zürich, Switzerland,

Campiglia E. [c], Radicetti E. [c] Mancinelli R. [c]

[c] Department of Agricultural and Forestry Sciences, Tuscia University, Via S. Camillo de Lellis snc, 01100, Viterbo, Italy

Fradgley, N., [d]

[d] NIAB, Huntingdon Road, Cambridge, CB3 0LE, UK.

Pearce, B. [e],

[e] The Organic Research Centre Elm Farm, Hamstead Marshall, Newbury, Berkshire, RG20 0HR

Bergkvist G. [f],

[f] Department of Crop Production Ecology, Swedish University of Agricultural Sciences, box 7043, 750 07 Uppsala, Sweden

Finckh M.R.[g]

[g] Organic Agricultural Sciences – Ecological Plant Protection Group, University of Kassel, Nordbahnhofstraße 1a, 37213 Witzenhausen, Germany

*Corresponding author: marinari@unitus.it

Abstract

The goals of conservation agriculture are to preserve and enhance the soil resource base and the environment. Subsidiary crops (SCs), such as *Trifolium, Medicago, Vicia, Brassica*, *Raphanus* spp, are important components of conservation agriculture since they maintain the soil resource. However, the importance of SC species and environment on soil microbial communities are not well known. The overall objective of this study was to assess the effect of various subsidiary crops cultivation on soil microbial biomass and activity at four sites across Europe. The experiments were conducted during 2014 and 2015 at sites in the Nemoral (Sweden SLU), Oceanic (United Kingdom ORC), Continental (Switzerland AGS) and Mediterranean north (Italy UNI) pedo-climatic zones. The specific objectives were to determine: (i) the effect of SC growth on soil microbial biomass and activity, ii) the site-specific effect of SC growth on soil biochemical properties. The SCs consisted of leguminous or brassicaceous species sown after wheat harvest, or clover species under-sown in wheat. At 0-30 cm depth, microbial carbon and nitrogen increased under SCs at most sites indicating that SCs cultivation may favor soil biological fertility. Effects of SCs were similar in the pedo-climatic zones where air temperatures are never below 0 °C (ORC and UNI). Arylsulphatase was the most sensitive enzyme to legumes in the Mediterranean north (UNI). Chitinase activity was enhanced by SCs in the Oceanic and Nemoral pedo-climatic zones. High precipitation and the low average temperature, typical of Continental and Nemoral zones, may represent limiting factors for soil enzyme activity under all selected SCs. Among the four pedo-climatic zones, the Mediterranean north represented the most suitable environment to promote SC growth and soil coverage. This study showed that SC cultivation affects soil quality enhancing biochemical activity; however the SCs effect were influenced by the different pedo-climatic conditions.

Keywords: subsidiary crop; microbial biomass; specific enzyme activity.

Introduction

Subsidiary crops (SCs), such as *Trifolium, Medicago, Vicia, Brassica, Raphanus* spp are grown primarily for their agro-ecological services. Subsidiary crops can be non-leguminous species such as grasses (*Poaceae)* including cereals grown for that purpose, crucifers (*Brassicaceae*), other flowering plants, or legumes (*Fabaceae*). Leguminous species are widely used as SCs because of their ability to fix atmospheric nitrogen in symbiosis with Rhizobia. A large part of the N from legumes tends to be released soon after their suppression, as the residue decomposition process is generally rapid mainly due to their low C/N ratio (Radicetti et al., 2016). Moreover, legumes have a greater positive effect on soil microbial biomass than other species due to a higher root exudation rate (Chen et al., 2008).

The adoption of SCs, in combination with minimum soil tillage practices and within a well-planned crop rotation, is the main pillar of conservation agriculture (Creamer and Dabney, 2002; Pittelkow et al., 2015). Subsidiary crops protect the soil from erosion, in particular during the fallow period between the two main cash crops, and they provide a continuum of root systems in soil, promoting soil microbial biomass and its activity through rhizodeposition that provide uniform supply of organic C, as an energy source for microorganisms (Kumar et al., 2006; Paterson, 2013). The root system of SCs may increase soil microbial abundance and activity by enhancing the stabilization of soil macro-aggregates (Gyssels et al., 2005), which are ‘hot spots’ for soil microorganisms (Nannipieri et al., 2003; Sexstone et al., 1985). Some SCs with tap roots can penetrate deeply and help break up hard pans and bring nutrients up from deep layers while other SCs with fibrous roots, especially grasses, will increase soil carbon through their extensive root systems (Sarrantonio, 2012). Before cover crop suppression the presence of plant roots has a large impact on soil microbial communities and root exudates supply energy to soil microbes more efficiently than decomposing roots and crop residues (Calderon et al., 2016). In addition to their below ground effect, SCs can produce a large amount of above ground biomass. They promote nutrient cycling, and thus

77 soil fertility, particularly when they are incorporated into the soil as green manure (Mancinelli
78 et al., 2013; Fageria et al., 2005) or when they are mowed and left on the soil surface as
79 organic dead mulch (Hartwig and Ammon, 2002). The beneficial effect of SCs on soil is, thus,
80 the sum of all the above described aspects. However, most of the studies do not discriminate
81 among these different aspects, which contribute to the overall beneficial outcomes. Additional
82 knowledge may be obtained when assessing the effect on soil before SCs suppression during
83 their growth cycle; in this way it can be highlighted a specific effect due to root exudation and
84 relative products.

85 The impacts of SCs on soil nutrient biogeochemical cycling are usually documented in
86 relation to the soil organic carbon pool variation (Mukumbareza et al., 2016), which drives
87 soil microbial activity, inducing a priming effect of native soil organic matter (SOM) (Insam
88 and Domsch, 1988; Blagodatskaya and Kuzyakov, 2008; Murphy et al., 2011). Changes in
89 agronomic practices may cause long-term changes of the total soil organic carbon content
90 (Poeplau and Don, 2015). In the short-term, differences in soil C and N labile pools and soil
91 enzyme activities can be used as indicators of biological activity and they are widely used to
92 detect soil responses to agricultural management practices (Ramos et al., 2010; Zhou et al.,
93 2012). The greater soil microbial biomass and activity occurring after SC suppression
94 contribute to bio-geochemical nutrient cycling (Chavarria et al., 2016; Mbuthia et al., 2015).
95 In this context, soil biochemical properties as related to soil microbial activity are often used
96 as indicators of ecological changes and can be used to evaluate mineralization process
97 dynamics based on substrate availability and seasonal fluctuations (Mancinelli et al., 2013;
98 Marinari et al., 2015).

99 The benefits to the agro-ecosystem provided by SCs strongly depend on pedo-climatic
100 conditions (Mondal et al., 2015), land use intensity (Wittwer et al., 2017) and SC type
101 (Poffenbarger et al., 2015). These factors in turn affect crop productivity, the decomposition
102 rates of SOM, and the abundance of substrates that can be directly used by the soil microbes

(Davidson and Janssens, 2006; Marinari et al., 2015). Depending on SC species and pedo-climatic conditions SCs are likely to influence the biochemical properties differently. The short-term effect after SC suppression on soil properties (i.e. the joint effects of the above ground biomass and roots incorporation) has been widely investigated (Pérez-Álvarez et al., 2013; Marinari et al., 2015). Conversely, there is only little knowledge on the effect of SC growth on soil nutrient availability and microbial biomass and activity. The innovative aspect of this work was therefore to emphasize the beneficial effect of SCs growth, through their specific root system and products, on soil properties.

The overall objective of this study was to fill this gap by assessing the effects of various standing SCs on soil carbon and nitrogen labile pools, and microbial activity. It was hypothesized that soil biochemical properties are influenced by the growth of SC species and pedo-climatic conditions. Therefore, coordinated field experiments were conducted at four sites located in different pedo-climatic zones across Europe. The effects were studied at the end of the cropping cycle of the SCs before soil tillage. In particular, the aims of the study were: (i) to assess the short-term effect of SC root system before suppression on soil microbial biomass and its activity, in different pedo-climatic zones (ii) to assess how the annual meteorological conditions may interact with the short-term effect of SCs growth on soil biochemical properties.

2. Materials and methods.

2.1. Experimental setup and vegetation assessment

Field experiments were carried out in 2013/2014 (Cycle I) and 2014/2015 (Cycle II) in adjacent fields at four European sites (Figure 1). These sites represent a broad range of pedo-climatic zones (Jongman et al., 2006): Nemoral (Swedish University of Agricultural Sciences, hereafter called SLU), Atlantic Central (Suffolk, United Kingdom - Organic Research Centre,

ORC), Continental (Tänikon, Switzerland - Agroscope (AGS) and Mediterranean north (Viterbo, Italy - University of Tuscia, UNI). The pedo-climatic conditions at the four sites were quite different, which are summarized in Table 1 and Figure 1. The aridity index (AI) was reported in addition to annual average of rainfall and temperature. The AI was calculated on a monthly basis as:

$$\mathbf{AI} = \frac{\mathbf{P}i}{\mathbf{T}i + \mathbf{10}} \quad (1)$$

where AI = aridity index; Pi = monthly precipitation amount; Ti = monthly mean air temperature (Mancinelli et al., 2013). According to this index the wettest site (AGS), had the highest value in both Crop Cycles I and II; while the driest sites were ORC in the Cycle I and UNI in Cycle II. The SLU site had the highest organic carbon content (C_{org}) and an acid pH. The UNI had the lowest C_{org} content while ORC soil was the most alkaline.

All sites followed a common design starting with winter wheat (*Triticum aestivum* L. or *Triticum durum* Deff.) cultivated in the first year after seedbed preparation by ploughing and harrowing (Table 2). Wheat was sown either alone or intercropped with a leguminous species [subclover (*Trifolium subterraneum* L.) at UNI and AGS; white clover (*Trifolium repens* L.) at SLU and yellow trefoil (*Medicago lupulina* L.) at ORC] (Table 2). The leguminous species were chosen for their abilities, either to self reseed (subclover) or to re-grow (white clover and yellow trefoil), in order to act as cover crop after wheat harvest. Wheat alone was followed either by a SC sown immediately after harvest of wheat or bare (weedy) soil. The SCs sown after the wheat were either the legume hairy vetch (*Vicia villosa* L. at SLU, AGS and UNI) or *Brassica* based (Oilseed radish, *Raphanus sativus* L., at SLU and AGS and a *Brassica* mixture at ORC). A mixture of brassica and yellow trefoil was adopted as cover crop at ORC (Table 2). At each site, a bare soil without SCs was adopted as a control. In all experiments, the SCs treatments were replicated four times in a randomized complete block design. In both crop cycles, the percentage ground coverage of SC species was visually assessed at the end of

153 SC crop cycle. The soil coverage by the SCs differed among the four experimental sites. At
154 ORC, the brassica was partially killed due to frost over the winter. Similarly, at SLU the
155 oilseed radish and the hairy vetch were frost killed during winter. However, the hairy vetch
156 recovered to produce some biomass before being terminated (Figure 2).

157

158 *2.2. Soil chemical and biochemical properties*

159 Soil samples were collected at 0-30 cm depth before the establishment of the experiment in
160 order to define the initial soil properties at each site. The second sampling was conducted, at
161 the same depth, at the end of SC cultivation period, before preparation of seeding main cash
162 crop, in the spring of 2014 and 2015 for Cycle I and II, respectively. The 0-30 cm soil
163 sampling depth was established according to the soil volume mainly explored by the root
164 system. Three soil cores per experimental unit were taken using a 5 cm diameter auger, air-
165 dried before sieving (2 mm) and preserved at room temperature for no more than four months.
166 Soil moisture content of air dried sample was adjusted to 60 % of the water holding capacity,
167 and soil samples were then left to equilibrate in the dark at room temperature for 3 days prior
168 to measuring extractable C and N content, microbial biomass and enzyme activity. Total
169 organic carbon (C_{org}) and nitrogen (TN) contents were determined using the dry combustion
170 method with Thermo Soil NC—Flash EA1112 elemental analyser (Tiessen & Moir, 1993).
171 Each sample was pre-treated with a 10% HCl solution to eliminate carbonates. In order to
172 avoid differences induced by different levels of soil organic matter between sites, the soil
173 labile pool and microbial activity were expressed on soil organic carbon mass base. Microbial
174 biomass carbon (C_{mic}) and nitrogen (N_{mic}) were determined according to the fumigation–
175 extraction method (Vance et al., 1987), using the TOC-V CSN and TNM-1 analyzer
176 (Shimadzu, Japan). Microbial C (C_{mic}:C_{org}) and N index (N_{mic}:TN) were calculated as a
177 percentage of the total organic C and N. Extractable carbon (Extr C) and nitrogen (Extr N)

were determined using the same equipment on non-fumigated samples and expressed as a percentage of total organic C and N, respectively.

The following hydrolytic enzymes, known to be a part of soil biogeochemical cycles of C, N, P and S (Nannipieri et al., 2012), were analyzed: for carbon β-glucosidase (EC 3.2.1.21), α-glucosidase (EC 3.2.1.20), xylosidase (EC 3.2.2.27) and cellobiohydrolase (EC 3.2.1.91); for nitrogen chitinase (EC 3.2.1.30), for phosphorus acid-phosphatase (EC 3.1.3.2); for sulphur arylsulphatase (EC 3.1.6.1). Finally, the butyrate esterase (EC 3.1.1.1) was analysed as a proxy of intracellular activity (Wittman et al., 2004). Enzyme activities were determined using a microplate assay (Marx et al., 2001) with fluorogenic substrates (4-MUF-β-D-cellobioside, 4-MUF-N-acetyl-β-glucosaminide, 4-MUF-β-D-glucoside, 4-MUF-α-D-glucoside, 4-MUF-phosphate, 4-MUF-sulphate, 4-MUF-7-β-D-xyloside and 4-MUF-butyrate as substrates). Fluorescence (excitation 360 nm, emission 450 nm) was measured with an automatic fluorimetric plate-reader (Fluoroskan Ascent, Thermo Fisher Scientific, USA) and readings were taken after 0, 30, 60, 120 and 180 min of incubation at 30 °C (Marinari et al., 2013). The enzyme activities were expressed per unit of soil organic carbon (specific enzyme activities) (Trasar-Cepeda et al., 2008) in order to compare the sites that presented different organic carbon contents. Enzyme activities associate with the C cycle were expressed as Synthetic Enzyme Index (SEI C). The SEI C was calculated as the sum of 4 enzyme activities (β-glucosidase, α-glucosidase, xylosidase, cellobiohydrolase), which release the same reaction product in the microplate fluorometric assay (4-methylumbelliferone, MUF). The soil microbial functional diversity was calculated using the Shannon Diversity Index (H') calculated as:

$$H' = -\sum pi \ln pi \qquad (2)$$

where *pi* is the ratio of the activity of one enzyme to the sum of activities of all enzymes (Bending et al., 2002).

2.3 Data processing and statistical analysis

Analysis of variance (ANOVA) of chemical and biochemical soil properties data was performed separately for each Crop Cycle (I and II) and site. Fisher's protected least significant differences (LSD) at the 0.05 probability level ($P < 0.05$) were used for comparing the subsidiary crop treatments (leguminous CC, crucifers CC, living mulch and control). In addition, a two -way factorial experimental design was adopted for all parameters where the SCs was the main treatment and the year was considered as repeated measure (Cody & Smith, 1997), in order to verify the effects of annual meteorological conditions at each site. Normality of the data was checked using Kolmogorov-Smirnov test. The soil chemical and biochemical properties obtained in each experimental site were analyzed using Principal Component Analysis (PCA) in order to verify the effectiveness of the grouping variables to discriminate soil properties in the four pedo-climatic zones. This analysis was applied separately for each SC treatment. The statistical analyses were performed using the JMP 9.0 statistical software package (SAS Institute, Cary, NC).

3. Results

In order to compare the different sites across Europe a descriptive analysis, by means of the PCA, was performed. The PCA score plot showed that the soil properties at the four sites were generally separated regardless of SCs (Fig. 3). However, the clover treatments at ORC and UNI grouped together (Fig. 3c). Based on the loading factor (Table 1S – Supplemental material) the main soil properties discriminating between the pedo-climatic zones were the specific enzyme activities and the pool of labile nutrients, such as extractable and microbial carbon and nitrogen. In contrast, the C:N ratio of both soil and microbial biomass together with the microbial basal respiration contributed little to the groups separation. The highest

loading values on PC1 for each SC treatments and control soil were the specific enzyme activities (SEIC, Chit, Pho, Aryl). Moreover, the microbial carbon and nitrogen quotients C_{mic}:C_{org} and N_{mic}/TN, respectively can be included as loading factor for PC1 in case of clover living mulch and leguminous cover crops. Conversely, extractable C and N showed always high loading factors of PC2 for all treatments with positive coefficient in SC treatment and negative for control soil (Table 1S – Supplemental material). According to statistical analysis, when considering the year as a repeated factor in each site, most of the chemical and biochemical soil properties were affected by the field and annual meteorological conditions (data not shown). For this reason the effect of SCs, at 0-30 cm soil depth, was analyzed separately for each crop cycle.

Based on the site and cycle specific ANOVA, the microbial biomass and enzyme activities were the most sensitive soil indicators at 0-30 cm depth showing significant differences among SC treatments at each site (Table 3). For this reason the average values and the significant differences due to SCs have been shown in figures 4 and 5. Conversely, as expected, soil total organic C and total N did not change due to SC treatments at the considered soil depth. Moreover, the Extr C and Extr N, expressed per gram of soil were affected by SC treatment, while they were less sensitive when expressed per unit of organic carbon and total nitrogen, respectively (Table 3). Therefore, the range values recorded at each site have been showed in figure 6 to focus on variability due to pedo-climatic conditions. The SC treatment significantly increased the microbial carbon quotient (C_{mic}:C_{org}) at the Oceanic and Mediterranean north sites (ORC and UNI, respectively) in both crop Cycles. In the two years 2014 and 2015, similar SC effects were found on microbial nitrogen quotient (N_{mic}:TN) in the Continental and Nemoral sites (AGS and SLU, respectively). The C_{mic}:C_{org} was sensitive to brassica at ORC and to Vetch and subclover at UNI with higher values with respect to the control soil (Fig. 4a). Moreover, in the crop Cycle I at the AGS site, vetch showed the highest soil microbial carbon quotient. The microbial nitrogen quotient (N_{mic}:TN)

254 at AGS increased in all SC treatments compared to the control soil, while at SLU the brassica
255 promoted a high Nmic/TN at both crop Cycles I and II (Fig. 4b).

256 Several significant differences occurred at the Oceanic and Mediterranean north sites (ORC
257 and UNI) (Table 3). In particular, enzymes involved in C (SEIC), S (arylsulphatase) and N
258 (chitinase) cycles were affected by SC treatments (Table 3). Repeated effects across crop
259 cycles occurred only for chitinase activity in the northern sites (ORC and SLU) and for
260 arylsulphatase activity at the southern site (UNI) (Table 3). Other significant differences in
261 enzyme activity occurred between SC treatments with respect to chitinase an enzyme
262 involved in the carbon and nitrogen bio-geo-chemical cycle. The activity of this enzyme was
263 particularly enhanced in soil under vetch at the Nemoral site (SLU) and in soil under vetch
264 and subclover at the Mediterranean north site (UNI) (Fig. 5c). Moreover, the specific activity
265 of acid phosphomonoesterase was similar in all SC treatments at UNI and AGS while at ORC
266 the effect of brassica was significant only at the Cycle I and at SLU the effect of SC treatment
267 was evident only at the Cycle II. At UNI the specific activity of arylsulphatase was highly
268 positively affected by the leguminous SCs (Fig. 5d). As an overall observation, ORC and UNI
269 had the highest microbial biomass (C and N) (Fig. 5).

270 The percentage of extractable fractions to the total C and N content changed across the pedo-
271 climatic zones (Fig. 6a e b). Trends for extractable C and N, expressed as percentage to the
272 respective total amount of C and N, differed among sites (Fig. 6 a and b). While Extr C/C_{org}
273 tended to be similar among sites (Fig. 6a), Extr N/TN was highest in the Mediterranean north
274 and lowest in the Nemoral pedo-climatic zone (Fig.6b).

275 The enzyme specific activities varied between Cycles I and II as well as among the four pedo-
276 climatic zones as shown by the synthetic enzyme index (Fig. 7a). Moreover, microbial
277 functional diversity expressed by the Shannon index (H') was slightly higher at AGS
278 compared to the other sites (Fig. 7b). The ANOVA revealed that for H' differences along the

two cropping Cycles I and II were significant only at the Mediterranean north site UNI (Table 3) where SC treatments enhanced the microbial biomass functional diversity (Vetch 2.18 and subclover 1.98 vs. Control soil 1.90).

4. Discussion

In this study, the SC effect was observed at the end of SC cultivation period, before the preparation of planting bed of the following main cash crop. This innovative approach highlighted the combined effects of SC cultivation and pedo-climatic conditions on soil labile C and N pools and microbial activity over a two-year period. The majority of studies investigate the effects of SCs after their suppression and during the following main cash crop cultivation when the SC residues are either left on the soil surface, as dead mulch, or incorporated into the soil as green manure. This kind of approaches does not discriminate between the effects of SCs residues mineralization from the plant effects themselves such as rhizodepositions release (Radicetti et al., 2016; Marinari et al., 2015; Dinesh et al., 2001; Hu et al. 1997). SC suppression means a rapid and substantial input of organic matter usually during periods with climatic conditions conducive to microbial degradation processes. The usually slower and more variable processes of rhizodeposition and other microbial processes during SC growth, as influenced by pedo-climatic conditions, become, thus, indiscernible. Therefore, this work provides insight on soil biochemical changes induced by the species specific effects that SCs produce during their growth in relation to the root system depth (0-30 cm).

4.1 Effect of pedo-climatic zones on soil biochemical properties across Europe

As the two Cycles were conducted in different fields at the different sites, pedo-climatic and weather conditions cannot be fully separated. The interaction of pedo-climatic zones and SC species resulted in soil changes in terms of nutrient labile pool and biochemical activity and it was considered SC specific when the effect occurred in both Crop Cycles (I and II) although microbial population size and activity was shown to be very sensitive to seasonal fluctuation at UNI (Marinari et al., 2015). Therefore, the repeatability of the effects of SC growth on soil microbial biomass under different meteorological conditions suggests a dominant effect of SC on soil biochemical properties.

In the Mediterranean north and Oceanic pedo-climatic zones, where the aridity index (AI) was lower and temperatures were seldom below 0 ºC, the microbial indices (C_{mic}:C_{org}; N_{mic}:TN) and the synthetic enzyme index (SEI) were higher than in the Continental and Nemoral zones. This may be because soil microorganisms rapidly metabolize labile substrates when temperatures are mild (Davidson and Janssens, 2006; Marinari et al., 2015). Moreover, when the lowest temperature is combined with acidic soil pH, such as at SLU, the microbial pool is negatively affected with a lower C and N immobilization. Increased precipitation and temperature enhance the soil labile pool of C and N, which are easily decomposable by soil microorganisms and an important source of nutrients for the agro-ecosystems (Song et al., 2012). In this study, the effect of pedo-climatic conditions was particularly evident for Extr N/TN being the highest in the Mediterranean north zone. Moreover, in the Oceanic and Mediterranean north zones, effects of clover were almost equivalent making soils of the two pedo-climatic zones more similar compared to the other SCs and control treatments. This effect might be due to the fact that similar climate conditions (temperature never below 0 °C and low AI) allowed clover to grow more vigorously on soils characterized by differences in terms of physical and chemical properties. In this case, it can be supposed that climate was more effective to induce changes in Scs root system and, therefore, in soil biochemical properties. Conversely, spring-sown clover never died at SLU, but its growth was prevented

by the specific pedo-climatic conditions (e.g. low temperatures, acidic soil). Soil freezing has a direct influence on the soil water availability, causing a slower diffusion of C based substrates and enzymes within the soil matrix (Jefferies et al., 2010). Moreover, very cool or frozen soil either slows down microbial metabolism or may kill microorganisms due to cell starvation or rupture (Jefferies et al., 2010). Finally, in the Continental pedo-climatic zone (AGS) the soil specific enzyme activity was generally the lowest among all sites. Although, it is known that soil physical and chemical properties (Nannipieri et al., 2012) strongly affect soil enzyme activities, in this case we assume that the lowest activities found at AGS was due to the highest precipitation level observed in this site during SCs cultivation. Soil water *status* is an important aspect affecting microbial community activity (Pan et al., 2016) and structure since they are partly controlled by soil oxygen availability, which is in turn controlled by soil moisture (Drenovsky et al. 2004; Schimel et al. 2007).

4.2 Effect of SC cultivation on soil biochemical properties

Many studies highlighted short- and long-term beneficial effects of SCs on soil properties (Ruis and Blanco-Canqui, 2017; Sainju et al., 2000). Short-term studies are particularly interesting when they focus on microbial C and N dynamics, which respond rapidly to SCs incorporation and may thus provide important information on optimal dates for SCs incorporation and subsequent crop planting. Furthermore, when long term studies are not possible due to limitations imposed by scientific projects deadlines and widely distributed experimental site as in this case, short-term studies, using reliable bioindicators, may provide information on future trends on SOC turnover and/or sequestration (Ruis and Blanco-Canqui, 2017).

In this study, positive effects on soil biochemical characteristics associated to the biogeochemical cycles of nutrients, were observed after vetches and brassica-trefoil mixtures

353 cultivation than after brassica. The increase of soil biological activity observed in this study
354 may positively affect nutrient biogeochemical cycles, providing further beneficial effect to
355 crop production. Although in this study no data on root exudates were collected, it is likely
356 that the different effects of various SC species on microbial biomass pool and its activity,
357 could be due to the rhizosphere effect and root exudates occurring at 0-30 cm depth.
358 Moreover, it is known that the rhizosphere effect leads to changes in the composition, size and
359 activity of the soil microflora (Chavarria et al., 2016). In the Mediterranean north
360 environment (UNI), both leguminous SCs (vetch and subclover) resulted in an increase of
361 microbial biomass and their functional diversity (H') in both crop Cycles I and II. Enhancing
362 plant diversity increases soil microbial diversity; therefore populations of beneficial microbes
363 such as disease-suppressive bacteria can be increased by increasing plant functional group
364 richness including SCs in the crop rotation (Vukicevich et al., 2016). Similarly, in the
365 Continental environment (AGS) an increase of microbial nitrogen quotient (N_{mic}:TN) was
366 observed after vetch.

367 The significant positive effect on the soil microbial pool by leguminous SCs, especially vetch,
368 observed in the Mediterranean north and Continental pedo-climatic zones (UNI and AGS),
369 could probably be explained by the vigorous growth of the SCs, as indicated by the high soil
370 coverage, and the associated well-developed root system. Moreover, Leguminous species
371 have a great diversity of root exudates (Sugiyama and Yazaki, 2012) attracting a larger
372 amount of microorganisms compared with other SC families. This might explain the higher
373 microbial quotient and activity under vetch and subclover.

374 The short-term effect of SC due to fields and annual meteorological conditions was evident
375 when a different response of labile nutrient pools and enzyme activities was found between
376 the Crop Cycles I and II. Repeated positive effects (both Cycles I and II) on arylsulphatase
377 activity were found in the Mediterranean north environment. Conversely, in the Nemoral and
378 Oceanic pedo-climatic conditions (SLU and ORC sites), where SCs coverage was reduced

379 with respect to the other sites, a repeated positive effect (both Cycles I and II) of SCs was
380 registered for chitinase activity. The increase of arylsuphatase activity observed in soil under
381 vetch at UNI may suggest a high demand of S by this cover crop in the environment where its
382 growth is particularly enhanced. S in soil might promote root nodule growth and thus the
383 growth of legumes (Latef and Ahmad, 2015). Low concentrations of SO_4^{2-} in soils could
384 stimulate soil microbes to release arylsuphatase as extracellular enzyme (Saviozzi et al., 2006;
385 Wilhelm, 2009).

386 In the Nemoral and Oceanic climatic zones most of the specific enzyme activities were
387 sensitive to annual conditions, with the exception of chitinase activity. It is known that soil
388 nitrogen availability usually increases under legumes SC (Radicetti et al., 2016). This in turn
389 may act as a negative feedback on soil chitinase activity (Olander and Vitousek, 2000) while
390 promoting activity of other soil enzyme activity, such as phosphatase (Olde Venterink, 2011).
391 Moreover, the increase of chitinase activity can be attributed to the constant presence of
392 fungal populations in soils (Vepsäläinen, 2012) that could be particularly high in soil under
393 SC's in Nemoral and Oceanic pedo-climatic zones. Conversely, in Mediterranean pedo-
394 climatic zone, mild temperatures may promote bacterial community growth characterized by a
395 faster metabolism than other microbial groups (Pietikainen et al., 2005). Moreover, in the
396 same pedo-climatic zones, brassica SC had a positive effect on soil microbial C and N pools.
397 Previous studies reported that the size of microbial C and N pools are not only affected by
398 climate and crop but also by the growth stage of the brassicas with a higher biomass around
399 stem elongation (Sabahi et al., 2010). Therefore, even if the SCs at ORC and SLU sites did
400 not achieve complete soil coverage, the root system developed at 0-30 cm soil depth at the
401 end of the cover crop cycle may have promoted soil microbial biomass and activity. Finally,
402 the increase of C_{mic}:C_{org} observed at all sites suggests a carbon immobilization process within
403 the microbial biomass. This may lead to a positive future trend on soil C storage due to SCs.
404 C_{mic}:C_{org} is an early and sensitive predictor of changes occurring in soil organic matter

making it a useful parameter for short-term studies that do not allow the assessment of C_{org} changes (Marinari et al., 2006; Lagomarsino et al., 2009). The lack of significant effect of SCs on the carbon labile fraction (Extr C/C_{org}) supports the efficacy of this indicator suggesting that no losses as soluble carbon forms occurred.

5. Conclusions

In conclusion, SC cultivation positively affected, in the short-term, soil microbial biomass and activity. A dominant effect of SCs, independent of annual meteorological conditions, was found within climatic zones.

Among the four pedo-climatic zones, the growth of legumes as living mulch enhanced soil specific enzyme activity producing similar effects in Mediterranean north and Oceanic pedo-climatic zones. Moreover, the Mediterranean north was the most suitable to promote growth and coverage of leguminous SCs, enhancing soil microbial activity and functional diversity. As for Continental and Nemoral zones, the high precipitation level and the low average temperature, respectively represented limiting factors for soil enzyme activity under all selected SCs.

Subsidiary crops utilization was confirmed to be an effective agricultural practice enhancing soil biochemical properties. In this study it was found a sensitive short-term response of microbial biomass and activity even before SCs suppression. However, it is important to evaluate their potential beneficial effects in relation to the specific pedo-climatic area where the positive effects may have different extent.

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Table

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Table 1. Pedo-climatic description of the four European experimental sites. Corg = total organic carbon, TN = total nitrogen. For sites acronyms see section of Material and Methods.

Crop cycles	Sites	Rainfall (avg mm y^{-1})	Temperature (avg °C y^{-1})	AI	Soil order (USDA)	Clay (%)	Silt (%)	Sand (%)	Soil texture (USDA)	pH (H_2O) 1:2.5 w:v	Total carbonate (%)	Corg (g kg^{-1})	TN (g kg^{-1})
I (2013-2014)	SLU	598	8.2	2.66	Inceptisol	16	64	20	Silt loam	5.7	1.4	30.8	2.8
	ORC	628	10.8	1.19	Vertisol	58	20	22	Clay	7.5	4.6	20.7	2.0
	AGS	1111	9.5	3.74	Alfisol	19	35	46	Loam	7.1	1.0	20.1	2.2
	UNI	845	11.6	2.84	Entisol	23	22	55	Sandy-Clay-loam	6.7	0.4	11.0	1.1
II (2014-2015)	SLU	526	7.3	1.18	Inceptisol	21	64	15	Silt loam	6.1	3.0	28.6	2.2
	ORC	662	10.5	0.95	Vertisol	58	20	22	Clay	7.2	2.8	23.2	2.7
	AGS	1259	10.6	4.80	Alfisol	22	35	43	Loam	6.9	4.5	21.6	2.3
	UNI	614	11.2	0.86	Entisol	15	22	63	Sandy-loam	6.7	1.0	12.1	1.1

*Averages of rainfall and temperature are calculated considering data record of 12 months before soil sampling date, AI= aridity index is calculated on a monthly basis (30 days before soil sampling).

** Soil sampling at 0-30 cm depth

Table 2. Description of experimental set-up of crop cycle I and II. Each treatment were replicated four times.

Crop cycle	Sites	Crop and tillage history	Tillage	Main Crop	Subsidiary Crops treatments	Date of SCs sowing	Experimental unit size	Soil sampling date[1]
I	SLU	Arable land under conventional management with crop rotation (pre-crop: Winter wheat)	Plough (25-30cm depth) Harrowing [2] (5-10 cm depth)	Soft Wheat	Oilseed radish Hairy vetch White clover [3]	14.08.13 14.08.13 16.05.13	108 m^2 (9 m x 12 m)	05.05.14
	ORC	Arable land under organic management with crop rotation. Pre-crop was 5 years lay and prior to that potato.	Plough (25-30cm depth) Harrowing [2] (5-10 cm depth)	Soft Wheat	Brassica mixture[4] Brassica+Yellow trefoil Yellow trefoil [3]	21.08.13 21.08.13 03.05.13	24m^2 (2m x12m)	03.04.14
	AGS	Arable land under conventional management with crop rotation (pre-crop: pea)	Plough (25-30cm depth) Harrowing[2] (5-10 cm depth)	Soft Wheat	Oilseed radish Hairy Vetch Subclover [3]	21.08.13	48 m^2 (6 m x 8 m)	19.05.14
	UNI	Arable land under conventional management with crop rotation (Barley and previously a 2-year crop rotation of Durum wheat –Sunflower)	Plough (25-30cm depth) Harrowing[2] (5-10 cm depth)	Durum Wheat	Hairy Vetch Subclover [3]	15.09.14	22 m^2 (4m x 5.5m)	24.04.14
II	SLU	Arable land under conventional management with crop rotation (pre-crop: Spring oilseed rape)	Plough (25-30cm depth) Harrowing [2] (5-10 cm depth)	Soft Wheat	Oilseed radish Hairy vetch White clover [3]	25.08.14 25.08. 14 15.04. 14	72 m^2 (6 m x12 m)	05.05.15
	ORC	Arable land under organic management with crop rotation. Pre-crop was ley for 4 years that was preceded by winter wheat	Plough (25-30cm depth) Harrowing [2] (5-10 cm depth)	Soft Wheat	Brassica mixture[4] Brassica+Yellow trefoil Yellow trefoil [3]	06.07.14 06.07.14 18.03.14	24m^2 (2m x12m	10.04.15
	AGS	Arable land under conventional management with crop rotation (pre-crop: pea)	Plough (25-30cm depth) Harrowing [2] (5-10 cm depth)	Soft Wheat	Oilseed radish Hairy Vetch Subclover [3]	07.08.14	48 m^2 (6 m x 8 m)	02.06.15
	UNI	Arable land under conventional management with crop rotation (Barley and previously a 2-year crop rotation of Durum wheat –Sunflower)	Plough (25-30 cm depth) Harrowing [2] (5-10 cm depth)	Durum Wheat	Hairy Vetch Subclover [3]	21.09.15	22 m^2 (4 m x 5.5m)	29.04.15

[1]At the end of crop cycle; [2]Before each crop sowing; [3]living mulch; Forage rape, White mustard and Fodder radish

Table 3. Statistical analysis (ANOVA) of SCs effect on soil chemical and biochemical properties at the end of SCs crop cycle in the four European experimental sites (* p<0.05; ** p<0.01; *** p<0.001).

	SLU		**ORC**		**AGS**		**UNI**	
	Cycle I	Cycle II	Cycle I	Cycle II	Cycle I	Cycle II	Cycle I	Cycle II
Corg								
TN								
Extr C				***		*		*
Extr N			*			***	***	***
Extr C/Corg						**		
Extr N/TN					**			**
Cmic		***	***	**	***		***	**
Cmic:Corg		**	***	*	**		***	**
Nmic	*	***	**	*	*	*		
Nmic/TN	*	***	**		*	***		*
Cmic:Nmic		***	**		*	*	*	
SEI/Corg			***					*
SEIC/Corg			***					
Chit/Corg	**	*	***	*				**
Pho/Corg		*	*					
Aryl/Corg			**				**	***
H’							*	*

Corg = total organic carbon, TN = total nitrogen, Extr C= extractable carbon per gram of soil, Extr N = extractable nitrogen per gram of soil, Extr C/Corg = extractable carbon as percentage to total organic carbon, Extr N/TN = extractable nitrogen as percentage to total nitrogen, Cmic = microbial biomass carbon, Cmic: Corg =microbial quotient, Nmic = microbial biomass nitrogen, Cmic:Nmic = carbon to nitrogen ratio of microbial biomass, SEI/Corg = synthetic index of 15 enzymes activity per unit of organic carbon, SEIC/Corg = synthetic index of enzymes activity involved in carbon cycle, per unit of organic carbon, Chit/Corg= chitinase activity per unit of organic carbon, Pho/Corg= acid phosphatase activity per unit of organic carbon, Aryl/Corg= arylsulphatase activity per unit of organic carbon, H’ = Shannon diversity index.

Supplemental material

Table 1S. Loading factor values of the PCA related to figure 3; leguminous spp. (A), brassicaceous sp (B), clovers (C) and control plots (D). C/N = carbon to nitrogen ratio, Cmic:Corg = microbial carbon to total organic carbon ratio, Nmic:TN = microbial nitrogen to total nitrogen ratio, Ext C/Corg = extractable carbon to total organic carbon ratio, Ext N/TN = extractable nitrogen to total nitrogen ratio, Cmic/Nmic = microbial carbon to microbial nitrogen ratio, SEIC/Corg = sum of C cycle enzymes to total organic carbon ratio (C cycle enzymes specific activity), Chit/Corg = chitinase activity to total organic carbon ratio, Pho/Corg = phosphatase activity to total organic carbon ratio, Aryl/Corg = arylsulphatase activity to total organic carbon ratio, H' = Shannon diversity index.

	A		**B**		**C**		**D**	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
C/N	0.7	-0.3	0.6	-0.6	0.3	-0.7	0.1	0.8
Cmic:Corg	0.5	0.6	-0.3	0.9	0.5	0.7	0.2	-0.4
Nmic/TN	0.3	0.8	0.4	0.8	0.8	0.4	0.9	-0.2
Ext C/Corg	0.0	0.8	-0.6	0.7	0.1	0.7	0.4	-0.7
Ext N/TN	0.2	0.8	-0.6	0.5	0.1	0.8	0.4	-0.7
Cmic/Nmic	0.7	0.0	-0.8	0.1	-0.3	0.2	-0.6	0.2
SEIC/Corg	0.8	0.0	0.7	0.6	0.9	0.0	0.9	0.2
Chit/Corg	0.8	-0.2	0.7	0.7	0.9	-0.1	0.8	0.3
Pho/Corg	0.8	-0.1	0.9	0.1	0.6	-0.7	0.6	0.7
Aryl/Corg	0.6	0.1	0.6	0.0	0.5	-0.2	0.1	0.5
H'	-0.6	0.5	-0.4	-0.6	-0.7	0.0	-0.6	-0.3

Figure captions

Figure 1. Localization and weather conditions (monthly average of the daily temperatures and monthly total amount of rainfall) during the field experiments in 2013/2014 and 2014/2015 years of the four experimental sites.

Figure 2. Soil coverage of the SC treatments evaluated at the end of SC crop cycle: brassica sp. (Br), brassica and yellow trefoil mixture (Br YT), vetch (V), subclover (Sub C), white clover (Wh C), and control (C). Pedo-climatic zones: SLU (Nemoral); ORC (Oceanic); AGS (Continental); and UNI (Mediterranean north). Crop cycles I (dark grey) and II (light grey). Bars represent the standard error (n=4).

Figure 3. Principal component analysis (PCA) of the four SC treatments: annual leguminous (a), brassicaceous crops (b) sown after harvest of wheat, clovers under-sown in wheat (c), and control soil (d) over both crop cycles. The markers representing the experimental sites were: circles-AGS (Continental); squares-ORC (Oceanic); triangles-SLU (Nemoral) and asterisk UNI (Mediterranean north). For acronyms see table 3.

Figure 4. Soil microbial carbon (Cmic:Corg) (A) and nitrogen (Nmic/TN) (B) quotients after SC treatments determined at 0-30 cm depth: brassica spp. (Br), brassica and yellow trefoil mixture (Br YT), vetch (Ve), subclover (Sub C), white clover (Wh C), and control (C), across the four pedo-climatic zones SLU (Nemoral), ORC (Oceanic), AGS (Continental), and UNI (Mediterranean north), for crop cycles I (dark grey) and II (light grey). Values for each crop cycles and in each pedo-climatic zone without common letters are statistically different while asterisks indicate significant differences between crop cycles, according to LSD ($P < 0.05$).

Figure 5. Soil enzyme activities involved in biogeochemical cycles and their functional diversity determined at 0-30 cm depth: (A) C-cycle enzyme activities (SEI C); (B) chitinase (Chit), (C) acid phosphomonoesterase (Pho), and (D) arylsulphatase (Aryl). The activities are expressed per unit of

soil organic carbon. The CC treatments considered: brassica sp. (Br), brassica and yellow trefoil mixture (Br YT), vetch (V), subclover (Sub C), white clover (Wh C), and control (C) in the four pedo-climatic zones: Nemoral (SLU), Oceanic (ORC), Continental (AGS) and Mediterranean north (UNI) at both crop cycles I (dark grey) and II (light grey). Values for each crop cycles and in each pedo-climatic zone without common letters are statistically different while asterisks indicate significant differences between crop cycles, according to LSD ($P < 0.05$).

Figure 6. Soil extractable C expressed as percentage of total organic carbon (Corg) (a) and soil extractable N as percentage of total nitrogen (TN) (b) across the four pedo-climatic zones: SLU (Nemoral); ORC (Oceanic); AGS (Continental) and UNI (Mediterranean north) in both crop cycles I (dark grey) and II (light grey). Middle line represents the median and whiskers the standard deviations (n=16). Soils were sampled at 0-30 cm depth.

Figure 7. Soil synthetic enzyme index (SEI) (a) and microbial functional diversity (H') (b) across the four pedo-climatic zones: SLU (Nemoral); ORC (Oceanic); AGS (Continental)and UNI (Mediterranean north) in both crop cycles I (dark grey) and II (light grey). Middle line represents the median and whiskers the standard deviations (n=16). Soils were sampled at 0-30 cm depth.

Figure 1

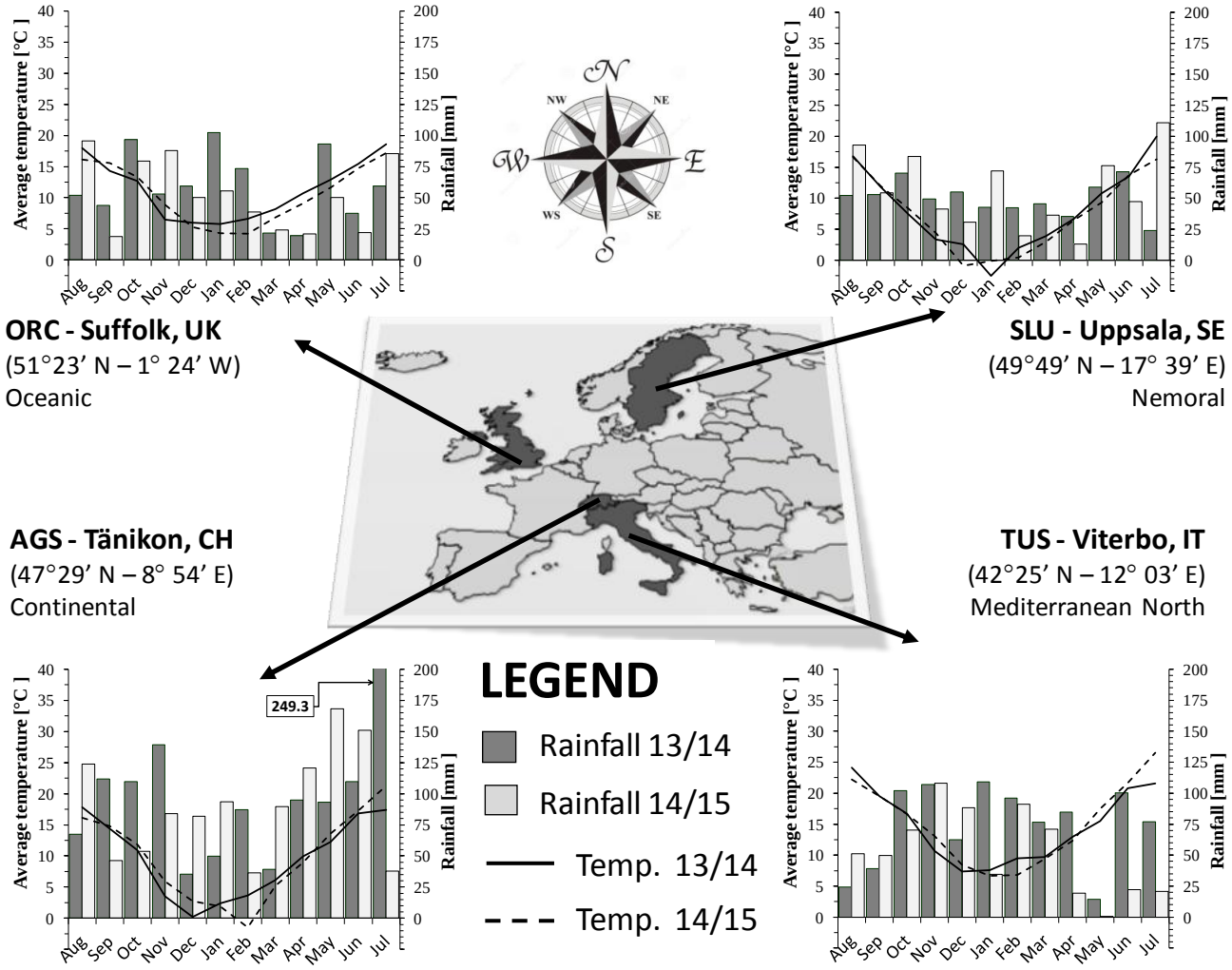

Average temperature [°C]
Rainfall [mm]
Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul
N
NW
NE
W
E
WS
SE
S
ORC - Suffolk, UK
(51°23' N – 1° 24' W)
Oceanic
SLU - Uppsala, SE
(49°49' N – 17° 39' E)
Nemoral
AGS - Tänikon, CH
(47°29' N – 8° 54' E)
Continental
TUS - Viterbo, IT
(42°25' N – 12° 03' E)
Mediterranean North
249.3
LEGEND
Rainfall 13/14
Rainfall 14/15
Temp. 13/14
Temp. 14/15

Figure 2

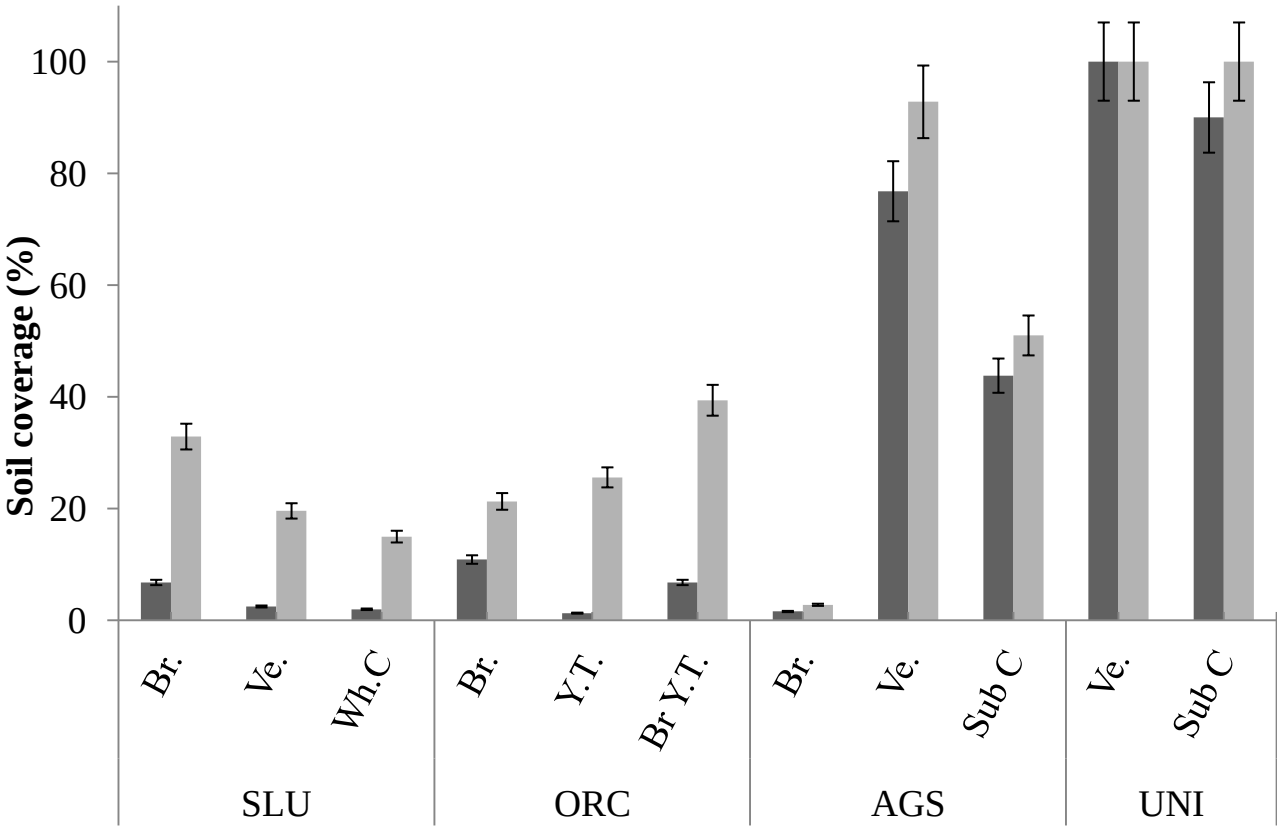

Soil coverage (%)
100
80
60
40
20
0
Br.
Ve.
Wh.C
Br.
Y.T.
Br Y.T.
Br.
Ve.
Sub C
Ve.
Sub C
SLU
ORC
AGS
UNI

Figure 3

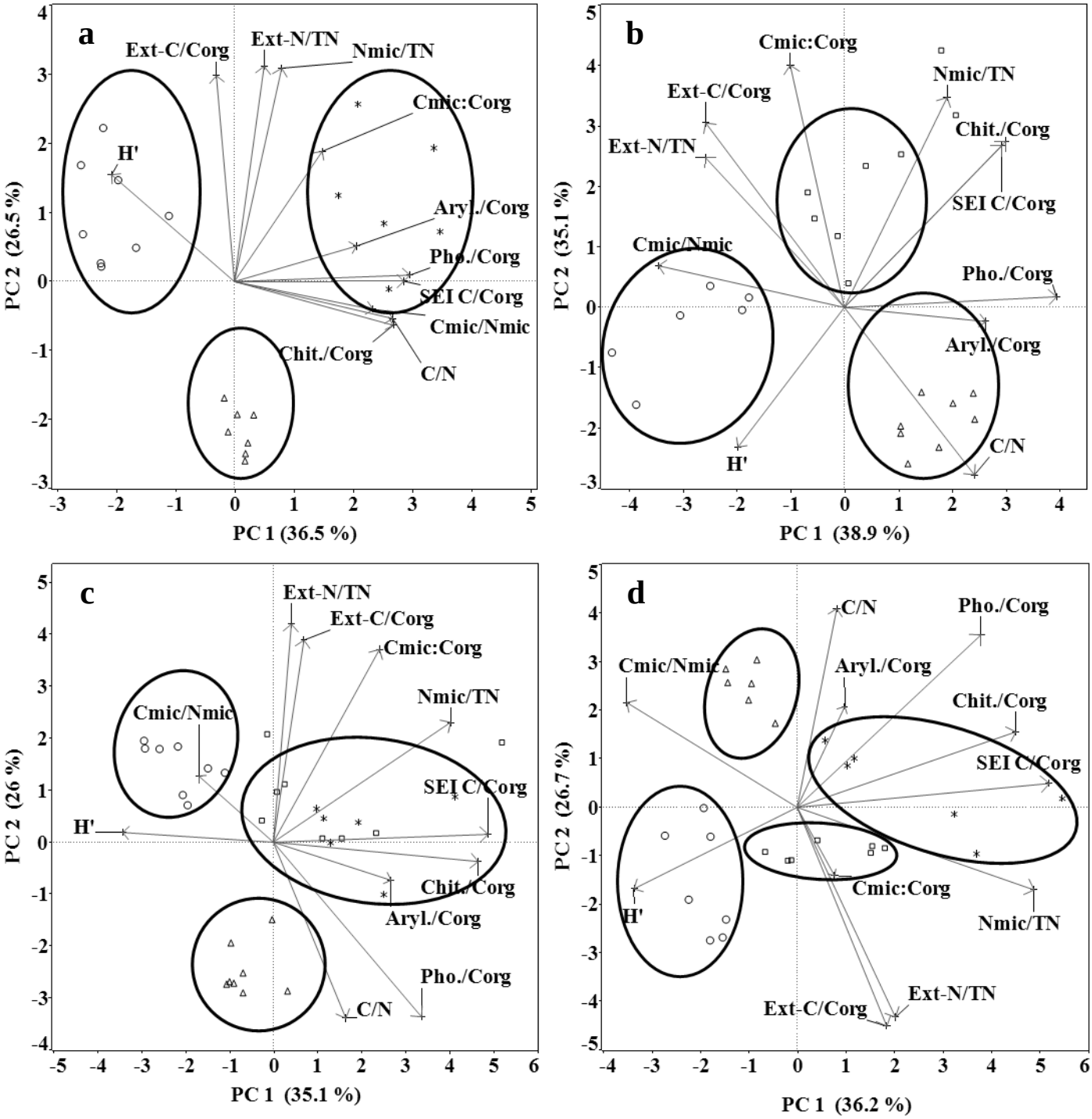

a
Ext-C/Corg
Ext-N/TN
Nmic/TN
Cmic:Corg
H'
Aryl./Corg
Pho./Corg
SEI C/Corg
Cmic/Nmic
Chit./Corg
C/N
PC 2 (26.5 %)
PC 1 (36.5 %)
b
Cmic:Corg
Nmic/TN
Ext-C/Corg
Ext-N/TN
Chit./Corg
SEI C/Corg
Cmic/Nmic
Pho./Corg
Aryl./Corg
C/N
H'
PC 2 (35.1 %)
PC 1 (38.9 %)
c
Ext-N/TN
Ext-C/Corg
Cmic:Corg
Nmic/TN
Cmic/Nmic
SEI C/Corg
H'
Chit./Corg
Aryl./Corg
Pho./Corg
C/N
PC 2 (26 %)
PC 1 (35.1 %)
d
C/N
Pho./Corg
Cmic/Nmic
Aryl./Corg
Chit./Corg
SEI C/Corg
Cmic:Corg
H'
Nmic/TN
Ext-C/Corg
Ext-N/TN
PC 2 (26.7 %)
PC 1 (36.2 %)

Figure 4

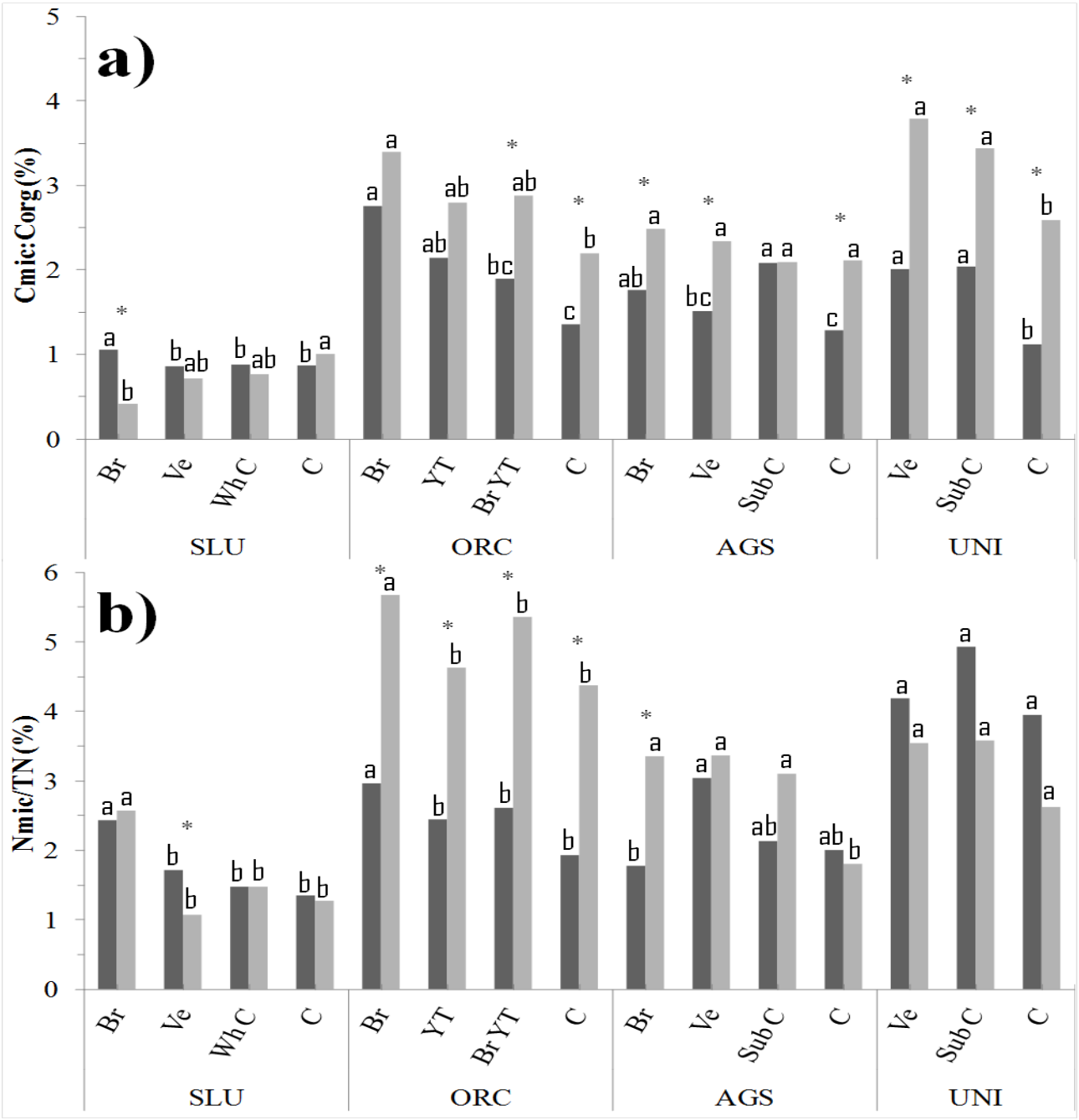

a)
Cmic:Corg(%)
0
1
2
3
4
5
Br
Ve
Wh C
C
Br
YT
Br YT
C
Br
Ve
Sub C
C
Ve
Sub C
C
SLU
ORC
AGS
UNI
b)
Nmic/TN(%)
0
1
2
3
4
5
6
Br
Ve
Wh C
C
Br
YT
Br YT
C
Br
Ve
Sub C
C
Ve
Sub C
C
SLU
ORC
AGS
UNI

Figure 5

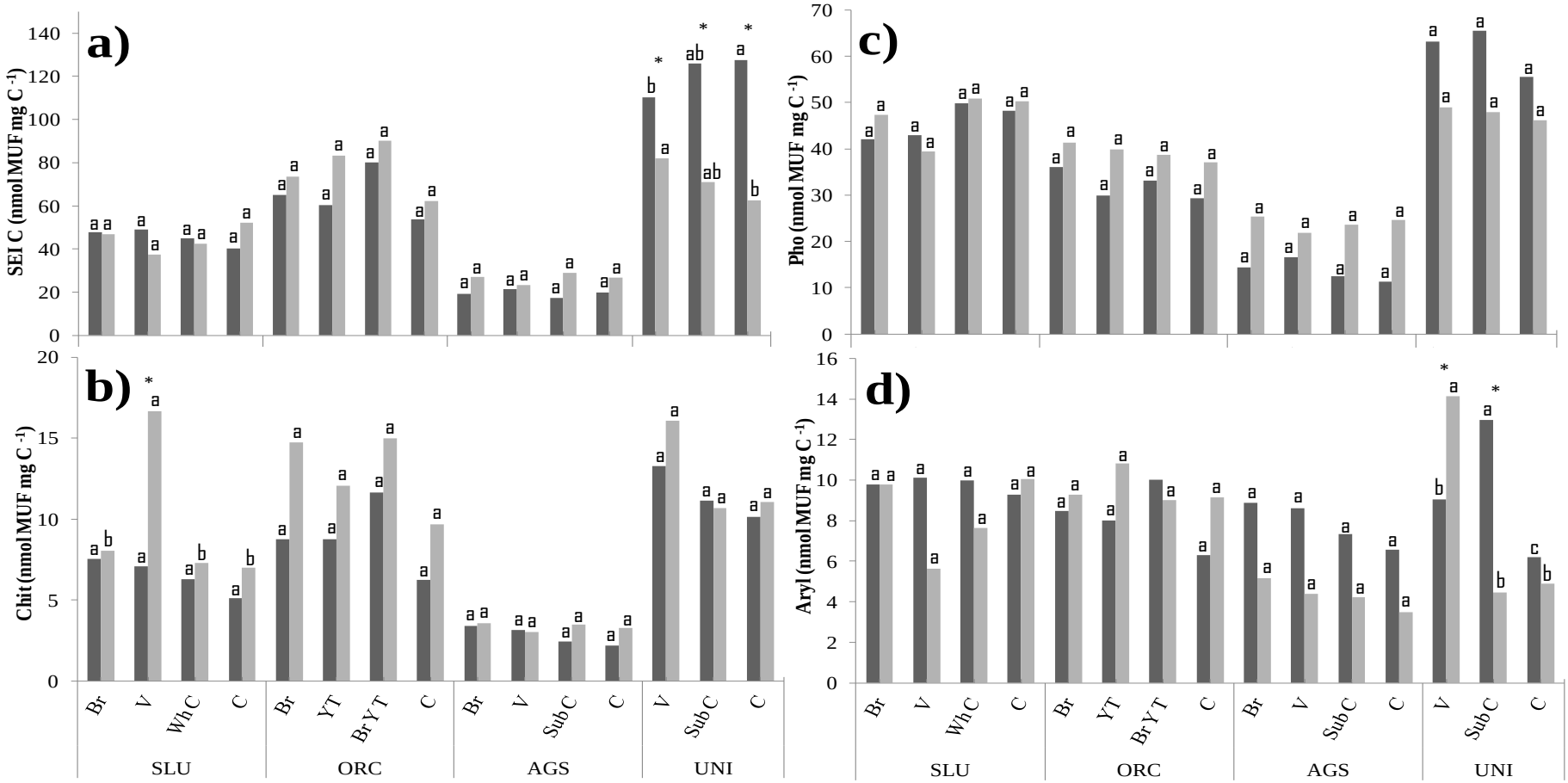

a)
SEI C (nmol MUF mg C -1)
b)
Chit (nmol MUF mg C -1)
c)
Pho (nmol MUF mg C -1)
d)
Aryl (nmol MUF mg C -1)
Br
V
Wh C
C
YT
Br Y T
Sub C
SLU
ORC
AGS
UNI

Figure 6

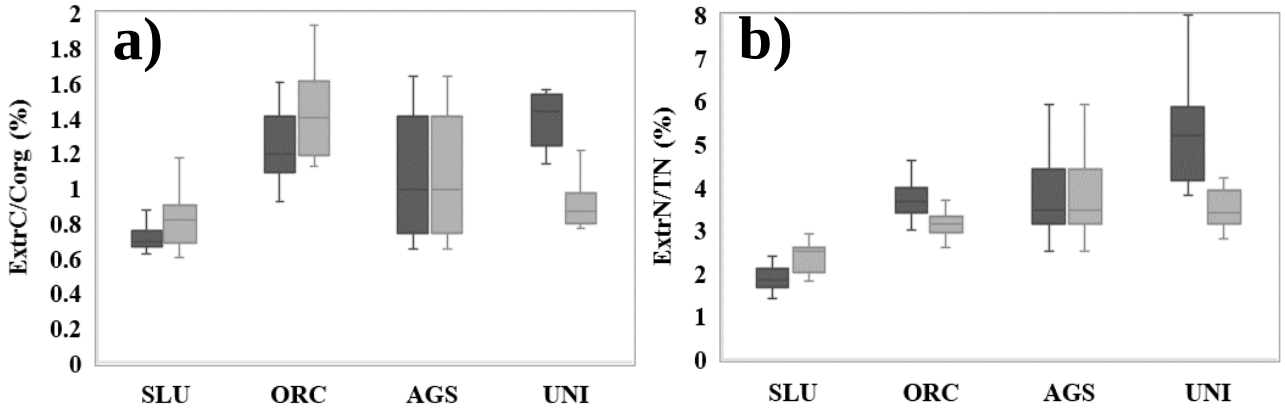
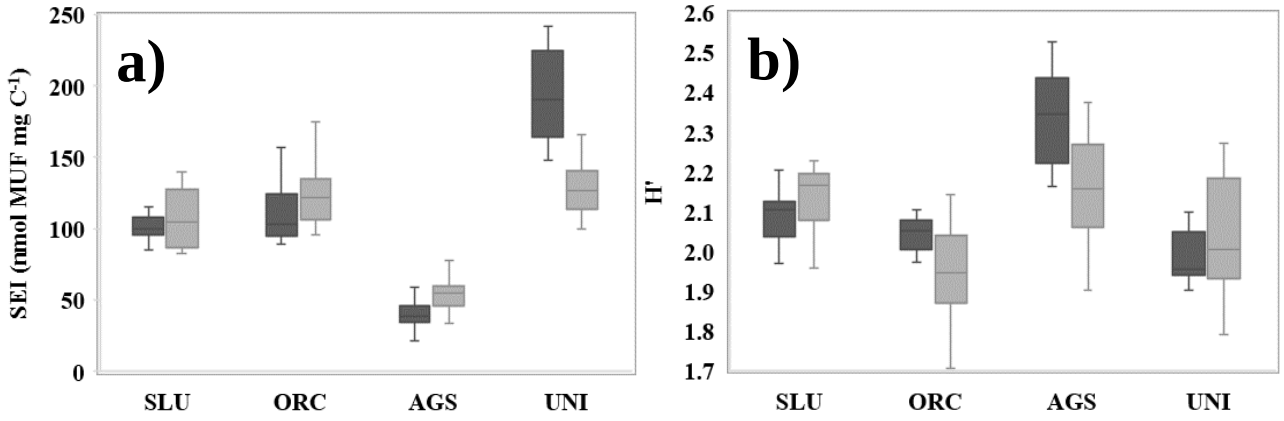

a)
ExtrC/Corg (%)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SLU
ORC
AGS
UNI
b)
ExtrN/TN (%)
8
7
6
5
4
3
2
1
0
SLU
ORC
AGS
UNI

Figure 7


a)
SEI (nmol MUF mg C-1)
250
200
150
100
50
0
SLU
ORC
AGS
UNI
b)
H'
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
SLU
ORC
AGS
UNI