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"CHANGES OCCURRING IN THE TOPSOIL AND IN RHIZOSPHERE UNDER FAGUS SYLVATICA ALONG A SMALL LATITUDINAL-ALTITUDINAL GRADIENT"

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Summary

1 Introduction

Soil is a complex system largely affected by climate (Aerts, 2006). Temperature is one of the climate's attribute, and its changing is one of the drivers of the soil physical, chemical and biochemical properties and functionality (Allen et al., 2011). Most of the studies on climate effect has dealt with the aboveground vegetation, plant diversity, or soil biochemistry (Hunt and Wall, 2002), but an extensive comprehension of the effect on soil functionalities is still poor, even if some studies have demonstrated the relationship between soil properties and climate change drivers. In particular, climatic stress such as warming has effect on the rhizosphere soil and topsoil, their microbial communities, and nutrient functioning.

The doctoral project focused on the investigation of the temperature effect on soil properties and processes. The project followed two main lines: *i)* the investigation of mineral soil, specifically of the rhizosphere effect on chemical soil properties, and *ii)* chemical and biochemical properties of the topsoil, which is formed by the organic horizons (litter and humus) and the first organo-mineral horizon in a gradient of decomposition.

The work on the organic horizons fell in a collaboration with the tem of prof. David Weindorf at the Texas Tech University of Lubbock (Texas, USA), where I developed an analytical method to estimate total carbon and total nitrogen with proximal sensors (PXRF[1] and VisNIR[2]) on organic soil materials. The use of these apparata has been already extensively documented for mineral soil horizons, but there was no information on their use on organic horizons.

1.1 The rhizosphere

1.1.1 Definition and general properties

The rhizosphere is the interface between plant roots and soil, where the interactions between abiotic and biotic factors create a zone distinct from the bulk soil, the soil not directly affected by living roots and microorganisms (Haynes, 1990; Philippot et al, 2013). Indeed, rhizosphere is the most important link between plants and soil, plays a central role in the maintenance of the soil-plant system and, in spite of the small volume that it occupies, it influences the biogeochemistry of natural and agricultural ecosystems (Gobran and Clegg, 1996; Gobran et al., 1998). The term *rhizosphere* is self-explanatory: *rhizo* comes from the Greek word "root" and *sphere* stands for the "environment" in which roots act (Gobran et al., 1998). The rhizosphere is usually subdivided into three ecological niches (Lynch, 1990):

[1] Portable X-Ray Fluorescence
[2] Visible Near InfraRed

- endorhizosphere, the thin layer that spans from the roots surface to the near surface cells, which is colonized or can be potentially colonized by microbes;
- rhizoplane, the external plant surface, namely the two-dimensional interface between root and soil;
- ectorhizosphere, the soil layer surrounding roots and affected by the activity of roots themselves, and the harboring microorganisms. The thickness of this portion usually ranges from one to a few millimeters.

In general, the term "rhizosphere" is used to indicate the whole environment between ecto- and endorhizosphere.

Rhizosphere effect

Ectorhizosphere and rhizoplane represent the major source of substrates for microbial activity due to their enrichment with rhizodeposition products (Lynch and Whipps, 1990). Rhizodeposition is the release of carbon compounds from living plant roots through the loss of organics from root epidermal and cortical cells that leads to a proliferation of microorganisms in this soil portion. Also root metabolite and root exudates are often released in large quantities into the rhizosphere from living root hairs or fibrous root systems (Bertin et al., 2003). Theoretically, almost any soluble component present inside the root can be released to the rhizosphere; however, evidences suggest that exudation is dominated by low molecular weight solutes such as sugars, amino acids and organic acids that are present in the cytoplasm at high concentrations (Farrar et al., 2003). Through the exudation of this wide variety of compounds, roots impact the soil microbial community in their immediate vicinity, influence resistance to pests, support beneficial symbioses, alter the chemical and physical properties of the soil, and inhibit the growth of competing plant species (Bertin et al., 2003). However, their production is substantially dependent on the soil and other environmental conditions, such as anoxia, mechanical force, water stress, nutrient status, temperature, pH and day-length (Cheng et al., 1993).

Another important process occurring at high extent in this soil fraction, and that is able to increase C release, is respiration. Functionally, rhizosphere respiration consists of root respiration, together with microbial respiration due to the use of root-derived materials (rhizodeposition) by microbes. The ecological implications of root respiration are different from those of root exudation. Root respiration consists of a direct release of photosynthetically-fixed C, whereas root exudation is a process through which photosynthetically-fixed C enters the C pool in the soil (Cheng et al., 1993). It is reported that 30–80% of the carbon translocated to the roots is lost through respiration.

Thus, root respiration is a major component of forest ecosystem carbon budgets (Lambers et al., 1995).

Both these processes, rhizodeposition and respiration, are responsible for generating the "rhizosphere effect".

Rhizosphere as driving factor of soil formation

It is documented (Misra et al., 1987; April and Keller, 1990; Shaffer et al., 1990; Hinsinger et al., 2005; Yong et al., 2006) that roots activity induces mechanical macro- and micro-modifications since it has the capability to change the physical properties of surrounding soil, producing chemical interactions that affect mineral weathering. These processes occur mainly through soil acidification induced by roots activities. Acidic root secretions were attributed to carbonic and organic acids produced by rhizosphere microflora and roots through respiration and exudation. Root and rhizosphere microbial respiration is thus expected to potentially contribute to change CO_2 concentration in soil, especially in alkaline soil whit the formation and stabilization of H_2CO_3 and to produce organic anions, and thereby soil pH (Hinsinger et al., 2003). Changes of pH around the roots are induced also by the release of H^+ or OH^- to compensate for an unbalanced cation–anion uptake at the soil–root interface (Hinsinger et al., 2003). All these processes has as consequence an induced effect on mineral weathering, or rather the phenomenon that results in the fragmentation, decomposition and dissolution of minerals due to the combined action of physical, chemical and biological processes (Gobran et al., 2005). There is no doubt that mineral weathering is an important phenomenon that supplies nutrients in forest soil, and it plays a central role in the biogeochemistry on terrestrial ecosystems due to its effect on the production of secondary minerals, buffering of acid inputs, and support capacity of mineral substrate. The evidence of the impact of rhizosphere on weathering in the root surrounded environment is reported in several studies. Rhizosphere and bulk soil usually differ for the relative abundance of some minerals, not in the mineral assemblage (Seguin et al., 2005). In fact, in the rhizosphere there is a greater amount of less weatherable minerals because the most easily weatherable minerals are more impacted by roots activity than resistant minerals such as K-feldspars or muscovite (Courchesne and Gobran, 1997). For example, the abundance of vermiculite was shown to increase in the rhizosphere as a result of biotite weathering (Adamo et al., 1998). Easily weathered minerals such as amphiboles and expandable phyllosilicates are generally more abundant in bulk than in rhizosphere. An explanation of this is that the weathering of the easily weatherable mineral in the rhizosphere is fostered by the depletion of cations that are absorbed by plants. For

example, K and Mg are plant macro-nutrients, but their abundance in soil is often low compared to plant needs. As the soil solution is progressively depleted of K by plant uptake, the interaction between the liquid and the solid phases requires a transfer of K from soil minerals. A source of relatively available K is the interlayer of phyllosilicates, and the removal of K from the 2:1 mineral structures to replenish the soil solution is one of the main reason for vermiculite formation (Seguin et al., 2005). Another explicative example of the environmental changes induced by plant roots is reported in Cocco et al. (2013). In the Marche region (Italy), soil genesis occurs from alkaline marine sediments and, being the *Erica arborea* an acidophilic species, soil alkalinity is the reason of its scarcity in the region. However, the species is present in some spots of the region because, once established in the upper decarbonated horizons, *Erica* progressively modifies the soil at depth through the activity of its roots and associated microorganisms.

1.1.2 Methods to study and sampling the rhizosphere

While it is rather simple to define what both the rhizosphere and the bulk soil are, it is difficult to physically collect rhizosphere soil. In fact, because of the lack of a morphological delimitation between rhizosphere and bulk, collection of rhizosphere samples is unavoidably rather subjective. To limit this problem, a number of non-destructive procedures have been devised to separate the rhizosphere in laboratory-cultivated plants (*in vitro*) or in nature (*in vivo*), according to the type of plants investigated.
In vitro rhizotrons or rhizoboxes are usually used.

Rhizotrons: are pots or cans made of plastic material filled with homogeneous soil substrates (calcined sand and clay). The pots, with different shapes and dimensions, are adopted to facilitate the viewing and the sampling of the roots. The homogeneous substrate facilitates rhizosphere separation but it is far from reality.

Rhizoboxes: similar to the pots of the rhizotrons, rhizoboxes are able to physically separate the soil directly in contact with the roots, with no limitations on circulation of solution thanks to porous membranes of steel or plastic material.

These methods are used especially for herbaceous plants. Furthermore, we are not able to reproduce natural growth conditions and there is also the risk that they may not accurately represent the variation of roots function changing depending on plant age. This is particularly true for arboreal species.

There are several procedures to sampling roots *in vivo*. For grasses and seedlings it is sufficient to collect the whole plant, while for arboreal or shrub-like plants the opening of a pedological profile is requested. This procedure gives the opportunity to sample rhizosphere by horizons, which are distinct physical, chemical and mineralogical soil environments. After a geomorphological and pedological survey, a soil profile is opened according to the most representative situation of the environment. Samples are collected in plastic bags and a preliminary separation between rhizosphere and bulk soil is made in the field. Only roots averaging from 0.5 mm to 1 cm of diameter are usually collected (Seguin et al., 2004; Corti et al., 2005). Once in the laboratory, to separate rhizosphere from bulk it is generally adopted a time-consuming procedure that considers the rhizosphere as the soil remaining strictly adhering to roots after a gentle shaking, while, on the contrary, the bulk as the soil detached during this operation (Cocco et al., 2013).

1.2 The topsoil: organic and organo-mineral horizons

1.2.1 Definition and general properties

The topsoil is strongly influenced by enrichment of organic matter and coincides with the sequence of organic (OL, OF, OH, H) and underlying organo-mineral horizons (A). Organic horizons are formed by dead organic matter (OM), mainly leaves, needles, twigs, roots and, under certain circumstances, dead plant materials such as mosses and lichens. This OM can be transformed in animal droppings following ingestion by soil/litter invertebrates and/or slowly decayed by microbial (bacterial and fungal) processes.

Roots excluded, following the rate of recognizable remains and humic component, organic horizons have been grouped into three diagnostic horizons, OL, OF and OH, which roughly correspond to the Soil Survey Staff (2010) Oi, Oe and Oa horizons, respectively. Suffixes are used to designate specific kinds of organic matter horizons then detailed into types. Definitions from *European Humus Forms Reference Base* (Zanella et al., 2011) are:

OL (Organic and Litter): horizon characterized by the accumulation of mainly leaves/needles, twigs and woody materials. Most of the original plant organs are easily discernible by naked eye. Leaves and/or needles may be discolored and slightly fragmented. Humic component amounts to less than 10% by volume; recognizable remains are 10% and more, up to 100% in non-decomposed litter. Two types of suffixes: n and v, with the following meaning:

- OLn for new litter (age < 1 year), neither fragmented nor transformed/discolored leaves and/or needles;
- OLv for old litter (aged more than 3 months), slightly altered, discolored, bleached, softened up, glued, matted, skeletonized, sometimes only slightly fragmented leaves and/or needles.

The passage from OLn to OLv depends on environmental characteristics and litter quality.

OF (fragmented): horizon characterized by the accumulation of partly decomposed litter, mainly from transformed leaves/needles, twigs and woody materials, but without any entire plant organ. The proportion of humic component is 10 to 70% by volume. Two types of suffixes: zo and noz, with the following meaning:

- OFzo for zoogenically transformed material (degradation is mainly lead by soil animals): 10% or more of the horizon volume, roots excluded;
- OFnoz for non-zoogenically transformed material (degradation is mainly lead by fungi or other non faunal process): 90% or more of the horizon volume, roots excluded.

OH (humus): horizon characterized by an accumulation of zoogenically transformed material, i.e. black, grey-brown, brown, reddish-brown well-decomposed litter, mainly comprised of aged animal droppings. A large part of the original structures and materials are not discernible, the humic component is more than 70% by volume. OH differs from OF horizon by a more advanced transformation due to the action of soil organisms.

A: the organo-mineral horizons are formed near the soil surface, generally beneath organic horizons. Colored by organic matter, these horizons are generally darker than the underlying mineral layer of the soil profile.

The transition through OLn, OLv, OF, OH and A may be seen as steps of decomposition process, since litter is reduced to its elemental chemical constituent until the mineral layer. The decomposition has a key role in natural ecosystem since it is the plant-soil link. Plants (producers) provide the organic carbon required for the functioning of the decomposer subsystem. The decomposer subsystem, in turns, breaks down dead plant material and indirectly regulates plant growth and community composition by determining the supply of available soil nutrients (Wardle et al., 2004). Summarizing, decomposition has a close relation with soil biota, and soil biota is affected, in addition to chemical properties of the soil, by thermal conditions (Ascher et al., 2012). According to the Allen's review (Allen, 2004), climatic effects on litter decomposition can induce changes in soil temperature and soil moisture that alter the rates of litter mass loss directly and at very short time-scales

because of the high sensitivity of biological processes to temperature and water availability. At longer time-scales, they can operate indirectly through the effects of warming on plant litter quality.

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2 Aims of the research

The introduction of the thesis gives the tool and the motivations that stimulated the research subject and explains the context. Particularly, the intent of the doctoral project was to study the effect of environmental conditions like temperature on rhizosphere, litter degradation, and organic matter evolution of a natural environmental from *in vivo* samples. The response of beech plants under P unavailability was deeply investigated through rhizosphere capability to produce organic root-exudations, enrich soil with organic carbon and easily degradable compound, and promote microbial activity. The more available fraction of organic matter (water extractable organic matter, WEOM) of rhizosphere and bulk soil was characterized for its content of sugars, soluble phenols, tannins and lignins. As these aspects are strictly linked to degradation process, we investigated the chemical properties of organic layers and the trends followed by enzymatic activities, as indicators of microbial production, during the evolution of litter until the A horizons.

All the above-mentioned aspects were studied under a perspective of rising temperature, assuming that the processes recorded at the altitude of 800 m may shift at 1000 m if the temperature will increase of 1°C. With this study we found that, other than the mean annual temperature, also changing of seasonal thermal excursions can probably affect soil properties. The pedo-ecological research was preceded by the development of an innovative method to obtain data such us total carbon and total nitrogen from a non destructive and less time-consuming device in order to develop rapid and in field analysis.

The body of the thesis includes four articles, presented in form of independent chapters, based on researches made during the period of PhD, whose results are specifically explained within each chapter.

Paper I

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Non-saturated soil organic horizon characterization via advanced proximal sensors

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ABSTRACT

The organic fraction of soils is critically important to soil health and optimal ecosystem functioning. Traditional analysis of soil organic horizons (O horizons) has been dependent upon laboratory-based instrumentation. Simultaneously, the use of proximal sensors such as portable X-ray fluorescence (PXRF) spectrometry along with visible near infrared diffuse reflectance spectroscopy (VisNIR DRS) has gained popularity for providing rapidly acquired spectral and elemental data useful for soil physicochemical property quantification. However, PXRF and VisNIR DRS have mostly been applied to the assessment of mineral soils. This preliminary study evaluated 136 organic laden soil samples (most aptly described as upland, non-saturated O horizons) using both laboratory based instrumentation (CN analyzer) and proximal sensors to evaluate total carbon (TC) and total nitrogen (TN). Results revealed that combining model outcomes using model fusion improved TC and TN prediction accuracies relative to using an individual instrument (PXRF or VisNIR DRS) or model averaging with improvements in root mean square error (RMSE) on the order of 10–47% and 10–67% for TC and TN, respectively. Partial least squares + random forest (PLS + RF) approaches emerged as the best model for predicting both TC and TN in organic laden soil samples. These results suggest that the strong predictive applications of proximal sensors extensively documented on mineral soils, may show similar promise for determination of a wide number of physicochemical properties on organic soil matrices, yet further exploration with a larger and more diverse dataset is recommended.

1. Introduction

Organic matter decomposition is a fundamental process for sustaining life on Earth (Gosz et al., 1976). The term soil organic matter (SOM) refers to all organic material in soil, from freshly deposited detritus or litter to highly decomposed, stable forms such as humic and fulvic acids (Stevenson, 1994). Organic matter cycling helps to maintain eco-system functionality as several ecological functions are correlated to the decay processes of the organic layers of forest soils. Indeed, decomposition and mineralization processes of organic residues affect nutrient cycling and induce the release of elements that represent the principal resources for plants and microbes (Berger et al., 2002; Berg and McClaugherty, 2008), such as macro- and micro-nutrients, and essential molecules for energy metabolism, photosynthesis, and membrane transport (Huttl and Schaaf, 1997). One of the main factors controlling the organic matter decomposition processes is the quality of the litter produced by plants (Ge et al., 2013). The specific chemical proprieties of the plant litter and its decay products, in turn, influence the underly-ing mineral soil (Wardle et al., 2004; Ball et al., 2014). Six et al. (2004) noted that the decomposition of SOM has an impact on several important soil properties as it improves soil aggregation (Bronick and Lal, 2005), enhances the activity of the soil microbial community (Ball et al., 2014; Carrillo et al., 2012; García-Palacios et al., 2013), and affects mineral weathering (Qafoku, 2015) and soil fertility (Kaiser et al., 2008). Thus, the knowledge of the characteristics and composition of SOM, and Current methods of SOM characterization are well established (Nelson and Sommers, 1996), but are largely laboratory based.

Abbreviations: SOM, soil organic matter; TC, total carbon; TN, total nitrogen; VisNIR-DRS, visible near infrared diffuse reflectance spectroscopy; PXRF, portable X-ray fluorescence.

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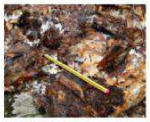
Table 1 - General description of the different types of organic horizons collected divided by study sites. For symbols see legend. a, b

Italy
Study site: Central Apennines - Mount Acuto, Mount San Vicino, and Mount Terminillo.

	Classification		Description
	Horizon[a] OLn	Horizon[b] Fibric	Easily recognizable beech cupules, leaves, twigs and bark. Thickness of 2 to 5 cm. Absence of tree roots and micelia. Very few and few presence of small macrofauna and mesofauna.
	OLv	Hemic	Brownish and degraded beech cupules, leaves, twigs and partially degraded bark and beechnuts. Thickness of 2 to 11 cm. General absence of tree roots; where present they are very few or few. Micelia is present from few to plentiful. Presence of small macrofauna and mesofauna from few to abundant.
	OH	Sapric	Extensive decomposition, plant parts are not recognizable. Darkish beechnuts. Reduced thickness of 1 to 4 cm. Dark horizon. Tree roots vary from absent to abundant. Micelia are generally abundant. Small macrofauna and mesofauna are plentiful to abundant.

Texas
Study site: Lubbock - North Fork of the Brazos River; Houston - George Bush Intercontinental Airport, WG Jones State Forest, San Jacinto River and Sam Houston National Forest.

	Classification		Description
Lubbock			
	Horizon[a] OLn	Horizon[b] Fibric	Organic layer originated by deposition after an alluvial event. Recognizable branches, leaves, with a predominance of twigs and bark. Spot with accumulation of grass leaves. Thickness of 4 to 6 cm. No roots and micelia. No small macrofauna or mesofauna activities.
	OLv	Hemic	Brownish and darkish degraded vegetal material made by not easily recognizable leaves, twigs, and bark. Thickness from 5 to 10 cm. Roots and micelia are not present. Very few and few presence of small macrofauna and mesofauna.
Houston			
	OLn	Fibric	Non-decomposed pine leaves, pine cones, twigs and bark. Thickness of 1 to 5 cm. Absence of root and micelia. Few presence of small macrofauna and mesofauna.
	OLv	Hemic	Brownish and pressed recognizable pine leaves, bark, and twigs. Thickness of 2 to 6 cm. General presence of tree roots from very few to few. Micelia generally goes from very few to abundant, with spots of very abundant presence. Considerable small macrofauna and mesofauna activities.
	OH	Sapric	Extensive decomposition, plant parts are not recognizable. Thickness of 1 to 2 cm. Tree roots vary from absent to few. Micelia is reduced from very few to plentiful. Small macrofauna and mesofauna are plentiful to abundant.

New Mexico
Lincoln County - Lincoln National Forest.

	Classification		Description
	Horizon[a] OLn	Horizon[b] Fibric	Not decomposed pine and deciduous leaves, pine cones, twigs, and bark. In spots, woody parts of bark from degradation of dead trees. Thickness of 1 to 7 cm. Absence of roots and micelia. The presence of degraded tree parts induces a considerable presence of small macrofauna and mesofauna, in general absent or few.
	OLv	Hemic	Degraded pine leaves and deciduous twigs, presence of pine cones. Degraded barks reduced in fine dust, structure not recognizable. Brownish horizons. Thickness of 1 to 8 cm. Few roots. Micelia generally goes from few to plentiful. Considerable small macrofauna and mesofauna activities.
	OH	Sapric	Vegetal material completely decomposed, only pine cones are still recognizable. Thickness from 2 to 3 cm. Generally few tree roots. Micelia is few to plentiful. Small macrofauna and mesofauna are plentiful to abundant

(continued on next page)

[a] Horizon designation per Association française pour l'Etude du sol (2008).
[b] Horizon designation per Soil Survey Staff (2014).

Recently, several studies have investigated rapid, inexpensive, and non-destructive methods, such as visible near infrared diffuse reflectance spectroscopy (VisNIR-DRS) and portable X-ray fluorescence spectrometry (PXRF) for soil analysis (Horta et al., 2015; Weindorf et al., 2014). These proximal sensing methods have become increasingly accurate and widely accepted offering data in situ in seconds given virtually no pre-processing requirements (Viscarra Rossel et al., 2006a, 2006b), with substantive advantages over traditional laboratory-based techniques. VisNIR-DRS is a spectrometric method which uses wavelengths across visible and near infrared regions (350–2500 nm) to explore the interaction between incident radiation and reflectance off of the soil surface; absorption is facilitated by C-H, N-H, or O-H bonds within the matrix (Chang et al., 2005). Due to this characteristic, it is highly applicable to C and N determination in soils. However, VisNIR-DRS spectra are generally weak, non-specific, and somewhat broad in their extent because of overlapping spectral signatures arising from variable soil components (Stenberg et al., 2010). As such, the instrument alone does not provide sufficient accuracy for complete soil characterization (Morgan et al., 2009). In fact, others have suggested the application of VisNIR-DRS in tandem with other sensing technologies (Brown et al., 2006; Fajardo et al., 2015). A complementary technique, PXRF, provides a multi-elemental analysis with a large range of quantification from low mg kg^{-1} to 100% for many elements (Hettipathirana, 2004). However, elements with stable electron configuration and low fluorescent energy (e.g., Na, N, H, Li, C) are not detectable (Wang et al., 2015). Nonetheless, several recent studies (e.g., Aldabaa et al., 2015; Chakraborty et al., 2015; Wang et al., 2015) have shown compelling predictive accuracy by combining the spectral signature of VisNIR-DRS with elemental data from PXRF, the latter used as auxiliary input data into the original advanced regression model. Individual or combined use of these two instruments allows for characterization of multiple soil parameters to include SOM (Stenberg et al., 2010), total carbon, total nitrogen (Wang et al., 2015), total phosphorus (Hu, 2013), cation exchange capacity (Sharma et al., 2015), pH (Sharma et al., 2014), salinity, (Swanhart et al., 2014), texture (Zhu et al., 2011), and contaminants (Chakraborty et al., 2015; Horta et al., 2015; Paulette et al., 2015). While the afore-mentioned studies offer wide-ranging application, most were conducted on mineral soils with limited organic content. Comparatively less information is available on the use of combined proximal sensors for soil organic layer (O horizon) characterization. Wang et al. (2015) evaluated total carbon and nitrogen via combined PXRF and VisNIR-DRS approaches, but did so on mineral soils, lacking any analysis of true organic horizons. Similarly, Chang and Laird (2002) have shown the efficacy of VisNIR-DRS to characterize soil carbon, but again, the soils evaluated were largely mineral soils. McWhirt et al. (2012) used a single sensor approach (VisNIR-DRS) to characterize the organic matter content of compost products. While organic, composted products differ substantively in their physicochemical composition from that of organic soils. By contrast, the present study explicitly aims to evaluate the combined use of both proximal sensors (PXRF and VisNIR-DRS) in characterization of organic soil horizons in variable states of decay. As such, the objectives of this study were to: 1) quantify total carbon and nitrogen in natural organic soils by VisNIR-DRS and PXRF individualistically and, 2) explore if there is a benefit in predictive accuracy from concatenating VisNIR-DRS spectra and PXRF elements. We hypothesize that total carbon and nitrogen of largely organic horizons can be directly predicted from the reported PXRF elements and VisNIR-DRS spectra. We further hypothesize that either a fused model or a model averaging approach will produce better predictability than either the VisNIR-DRS or the PXRF approach independently.

2. Materials and methods

2.1. General occurrences and features

In sum, 136 organic laden samples from non-saturated, uplands were collected in Italy and United States of America (Texas and New Mexico) during 2014 and 2015; a few mineral laden soils were also included as part of this dataset as a link to previously established work on mineral soils. The sites differed substantively in their geological composition, soil development, climate, and vegetation.

In Italy, a total of 39 organic horizons were collected from forest soils across three different sites on the Apennines chain (central Italy): Mount Acuto, Mount San Vicino, and Mount Terminillo. The soils developed from limestone of different geological origin: Mount Acuto is characterized by limestone (Lower Cretaceous - Aptian), Mount San Vicino is grey limestone with traces of flintstone and marl from the Jurassic (Lias) Pliensbachian Sinemurian and Mount Terminillo is grey limestone with trace amounts of flintstone (Jurassic Toarcian-Sinemurian) (ISPRA, 2015). The soils of these areas are classified as Mollisols or Inceptisols (Soil Survey Staff, 2014), characterized by a mesic soil temperature regime (10 °C to 12 °C) along with an udic soil moisture regime (from 825 mm to 1430 mm precipitation). In the three areas, the cover vegetation was mainly composed of *Fagus sylvatica* from 80 to 99%, with *Carpinus betulus* at Mount Acuto, *Quercus cerris*, *Castanea sativa*, and *Sorbus aria* at Mount San Vicino, and *Laburnum anagyroides* and *Acer* spp. at Mount Terminillo.

In Texas, 16 alluvial organic samples were collected in backwater areas along the North Fork of the Brazos River in Lubbock County in major land resource area (MLRA) 77C - Southern High Plains - Southern Part (Soil Survey Staff, 2006). Soils of this MLRA are generally developed by eolian deposits in the Blackwater Draw Formation of Pleistocene age, classified as Alfisols, Inceptisols, Mollisols, and Vertisols, and have a thermic soil temperature regime (13 °C to 17 °C) and an ustic soil moisture regime (from 405 to 560 mm precipitation). Mostly short and mid-prairie grasses and scanty tree and shrubs (e.g., *Bouteloua gracilis*, *Bouteloua dactyloides*, *Bouteloua curtipendula*) are prevalent. Separately, 27 various organic horizons were sampled in forested areas of the George Bush Intercontinental Airport, WG Jones State Forest, San Jacinto River, and Sam Houston National Forest; all generally in the vicinity of Houston, Texas. The WG Jones State Forest, San Jacinto River, and Sam Houston National Forest occur in MLRA 133B - Western Coastal Plain (Soil Survey Staff, 2006), where soils developed from Tertiary and Cretaceous marine sediments consisting of interbedded sandstone, siltstone, shale and loose primary particles. In particular, the Reklaw and Weches Formations in the Claiborne Group form the Redland area of East Texas. The main soil orders in this MLRA are Alfisols and Ultisols with a thermic soil temperature regime (16 °C to 20 °C), an udic or aquic soil moisture regime (990 to 1600 mm precipitation). Vegetation of the area is typified by pine-hardwood species such as *Pinus taeda*, *Pinus echinata*, *Liquidambar styraciflua*, *Quercus falcate*, and *Cornus flor-da*; *Callicapra americana*, and *Smilax* spp. are common in the woody understory. *Schizachyrium scoparium* and *Bothriochloa barbinodis* are the dominant herbaceous species. Of the 27 samples collected in this area, four were collected in densely wooded pine forests adjacent to George Bush Intercontinental Airport, which is a few kilometers beyond the aforementioned MLRA boundary, but quite similar in the organic horizons sampled.

In New Mexico, 54 samples were collected near the periphery of the Lincoln National Forest of Lincoln County; the horizons sampled were dominantly organic, but a few transitioned into organic laden mineral soils. The area is in MLRA 39-Arizona and New Mexico Mountains (Soil Survey Staff, 2006). The area is characterized by Cenozoic volcanic rock and various sedimentary sections of the Colorado Plateau. The southern and eastern parts contain Permian and Cretaceous sedimentary rock over a Precambrian granite core. Main soil orders of this MLRA are Inceptisols, Mollisols, Alfisols, and Entisols, with mainly frigid or mesic soil temperature regimes (2 °C to 13 °C) and an ustic/udic mois-ture regime (358 mm to 760 mm precipitation). *Pinus ponderosa* occurs is the dominant vegetation in the low and intermediate heights, while at higher altitudes, *Picea abies* and *Pseudotsuga menziesii* are commonplace.

2.2. Field sampling

For maintaining an extensive variation in terms of physicochemical characteristics, composition and origin, samples were randomly collected within morphologically established, organic laden horizons. Almost all samples were classified as O horizons according to Baize and Girard (2008). The different stages of degradation were classified as: i) OL, consisting of leaf debris with little or no degradation (b10% of fine or-ganic matter) whereby the botanic origin was easily recognized; sub-horizons were recognized as OLn, fresh litter with minimal degradation, and OLv, where the plant material was subject to initial degradation such as changes in color, volume, and inter-particle linkages; and ii) OH, with N70% of fine organic (humic) material following extensive decomposition (Table 1). The latter is homogenous, massive, and reddish-brown to black in color with an abundance of fine roots. These three states of decomposition are roughly akin to fibric, hemic, and sapric materials as defined by the Soil Survey Staff (2014), respectively.

2.3. Laboratory characterization and VisNIR scanning

Soil samples were air dried and ground to <1 mm prior to chemical analyses and spectral scanning. Total C (TC) and total N (TN) were determined by a TruSpec® dry combustion analyzer (LECO Corp., MI, USA) according to Dumas method combustion (Nelson and Sommers, 1996).

Spectral reflectance values of air dried and ground soil samples were measured proximally over the VisNIR region (350–2500 nm) by a portable PSR-3500® VisNIR spectroradiometer (Spectral Evolution, USA). The reflectance data were resampled to 1 nm output values. Scanning was done with a contact probe containing a 5 W halogen lamp, minimizing errors associated with stray light during measurements. Each sample was uniformly tiled in a glass petri plate and scanned four times, physically repositioning the probe prior to each scan. The mean of 10 internal scan over 1.5 s produced one individual scan. The spectroradiometer was standardized after every two samples using a NIST certified white reference. The average spectral curve was calculated and further used for spectral preprocessing and subsequent predictive modeling.

Necessary treatments of raw average reflectance spectra were done in R version 2.11.0 (R Development Core Team, 2008) following the spline fitting methods outlined in Wang et al. (2015). The spectral pre-processing involved converting reflectance to absorbance by log(1/R) which was executed in the Unscrambler®X 10.3 software (CAMO Soft-ware Inc., Woodbridge, NJ).

2.4. Scanning with PXRF

A DP-6000 Delta Premium portable X-ray fluorescence (PXRF) spectrometer (Olympus, Waltham, MA, USA) facilitated sample scanning. Configured with a Rh X-ray tube, the instrument was operated at 15– 40 kV; an integrated ultra-high resolution (165 eV) silicon drift detector quantified each element detected. Before scanning, a 316 alloy clip was used to standardize the instrument. Primary analysis was conducted in Soil Mode (three beams of 30 s each); it can detect the following: Ag, As, Ba, Ca, Cd, Cl, Co, Cr, Cu, Fe, Hg, K, Mn, Mo, Ni, P, Pb, Rb, S, Sb, Se, Sn, Sr, Ti, V, Zn, and Zr. A second scanning was performed with Geochem Mode (two beams of 30 s each) in order to measure Mg, S, Al, and Si. Geochem and Soil Mode scans were done in duplicate, with the spectrometer physically repositioned between each scan. Sample homogeneity was ensured during the grinding step. Data were then averaged between scans to obtain a mean of elemental data for each sample. Data quality was ensured via the scanning of two NIST certified reference samples, with recovery percentage calculated as PXRF reported vs. NIST certified values. Results were as follows (PXRF reported/NIST certified [recov-ery]): Zn 4263/4180 mg kg^{-1} [1.02]; Cu 3559/3420 mg kg^{-1} [1.04]; K 27,081/21,700 [1.25]; Ca 9119/9640 mg kg^{-1} [0.95]; Ti 3540/ 3110 mg kg^{-1} [1.14]; Mn 2237/2140 [1.05]; Fe 50,574/ 43,200 mg kg^{-1} [1.17]; As 1639/1540 mg kg^{-1} [1.06]; Sr 269/255 [1.05]; Pb 5608/5520 [1.02].

2.5. Data mining

We performed all statistical modeling via R version 2.11.0 (R Development Core Team, 2008) software. We also checked the normality of residuals by the Shapiro-Wilk test at a 5% significance level. In the present study, both original TC and TN values were negatively skewed (Pearson skewness coefficient −1.78 and −0.37 for TC and TN, respectively), while Box–Cox conversion (Box and Cox, 1964) using λ = 0 ($\log_{10}$ transformed) was unable to conform normal distribution. Principal component analysis (PCA) was executed via R version 2.11.0 using the 'prcomp' function to visualize spectral behavior of soil samples from different geographical areas and organic horizons. Optimal number of principal components (PC) was decided from a screeplot.

In this study, the whole dataset was split into 70% calibration (n = 96) and 30% validation set (n = 40) using the Kennard-Stone algorithm (Kennard and Stone, 1969), which is an adaptive procedure to select the most representative samples based on Euclidean distance. Calibration samples were used to establish a prediction model, whereas validation samples were used to assess the model's predictive ability (Chen et al., 2015). Initially, we targeted both TC and TN with VisNIR-DRS spectra only via partial least squares regression (PLS), elastic net regression (ENET), penalized spline regression (PSR), and random forest regression (RF) (Breiman, 2001; Guyon et al., 2002; Viscarra Rossel et al., 2006b). Additionally, both TC and TN were predicted with PXRF elemental data via PLS, ENET, and RF models.

Regularized methods, which play an important role in both statisti cal and data mining problems, can be described as Eq. (1):

$$\hat{\beta}(\lambda) = arg\ min_{\beta} L(Y, X\beta) + \lambda J(\beta) \quad (1)$$

where L(Y, Xβ) is a non-negative loss function, J(β) is a non-negative penalty on the model complexity and λ is the non-negative tuning parameter. The key idea for regularized methods is to balance between the goodness-of-fit on the training data and the complexity of the model. Usually, a complicated model always shows a good fit to the calibration data. Nonetheless, this commonly leads to problems of overfitting, where the model is too adapted to the training data and often has a poor prediction performance on new samples.

Elastic net, devised by Zou and Hastie (2005) is a regularized regression method that linearly combines the ridge penalty and the least absolute shrinkage and selection operator (LASSO) penalty (Eq. (2))

$$\hat{\beta}(\lambda_1 \lambda_2) = arg\ min_{\beta}\ SSE + \lambda_1 \sum |\beta_j| + \lambda_2 \sum \beta_j^2 \quad (2)$$

where SSE is the sum of squared errors, $\sum|\beta_j|$ is the LASSO penalty, and $\sum\beta^2_j$ is the ridge penalty. In high dimensional data analysis where many predictors are correlated to each other, the LASSO penalty tends to pick a few of the predictors and discard the others (sparse model), while the ridge penalty shrinks the coefficients of correlated predictors towards each other (dense model). The ENET penalty combines these two penalties so that when the predictors are correlated in groups, it will produce a sparse model with good prediction performance, while boosting a grouping effect. Another advantage of ENET is that it can handle the high dimension and low sample size problem. In this study, ENET was run using the 'glmnet' package in R, produced by Friedman et al. (2015). Details of PSR and RF methodology are summarized in Wang et al. (2015).

2.6. Model fusion and model averaging

Model fusion and model averaging (model ensemble) were tested to determine if combination of the predictions for the VisNIR-DRS and PXRF models into a single composite score could improve predictive accuracy of TC and TN. Initially, three fused modeling approaches (PSR + RF), (PLS + RF) and (ENET + RF) were employed where PSR/PLS/ENET were used to fit the training set (containing VisNIR-DRS spectra only). Next, we ran the RF using the PSR/PLS/ENET residual as the response and PXRF elements as the predictors (Chakraborty et al., 2015). Succinctly, the prediction value on the validation set using (PSR + RF), (PLS + RF) and (ENET + RF) contains two additive parts: the prediction from PSR/PLS/ENET model using the VisNIR-DRS spectra plus the prediction from RF model using the PXRF data. An outline of this fused procedure is shown in Fig. 1.

For model averaging we used Granger-Ramanathan averaging (GRA) (Granger and Ramanathan, 1984) with some modifications. The GRA approach requires fitting a multivariate linear regression model where lab measured soil property values are regressed against the corresponding predictions derived from VisNIR-DRS and PXRF models. Initially, the PSR/PLS/ENET model was fitted on the calibration set (n = 96) using the VisNIR-DRS spectra. Subsequently, the RF model was fitted using only PXRF elements as predictors and TC or TN as the response. Note that this RF model was not the same as the RF model in fused approaches (PSR + RF, PLS/RF, or ENET + RF) as the latter used residual as a response. Next, a bivariate linear regression was fitted with Eq. (3):

$$Y = a + b * X + c * Z \quad (3)$$

where Y is either TC or TN on calibration set; X is the fitted value (on calibration set) from the PSR/PLS/ENET model; Z is the fitted value (on the calibration set) from the RF model; a is the intercept; b is the estimated model weight for the PSR model; and c is the estimated weight for the RF model. To predict the validation set (n = 40), we first generated the prediction value, X*, from the PSR/PLS/ENET model, and prediction value, Z*, from the RF model. The GRA prediction is given as Eq. (4):

$$Y = a + bX^* + cZ \quad (4)$$

We followed the Kennard-Stone splitting scheme for both fused models and GRA.

2.7. Whole geographical area and organic horizon holdout validation

Further, whole geographical area and organic horizon holdout validations were executed for both TC and TN to determine how area to area and horizon to horizon heterogeneity affected prediction accuracies (Brown et al., 2005). Whole-geographical area holdouts were achieved by calibrating a model using the best performing algorithm with three geographical areas and then validated using the fourth area. Moreover, whole-organic horizon holdouts were achieved by calibrating a model using the best performing algorithm with two organic horizons and then validated using the third organic horizon.

In this study, the root mean square error (RMSE), regression coefficient (R^2), residual prediction deviation (RPD) (Eq. (5)), bias, and ratio of performance to interquartile distance (RPIQ) (Eq. (6)) were used to evaluate model performance (Gauch et al., 2003 Bellon-Maurel et al., 2010). RPD based model accuracy classification scheme devised by Chang et al. (2001) was followed for evaluating model accuracy.

$$RPD = \left[\frac{1/(n-1)\sum_{i=1}^{n}(Yabs - Ymean)^2}{1/n\sum_{i=1}^{n}(Yabs - Ypred)^2}\right]^{0.5}_{validation} \quad (5)$$

$$Bias = \sum_{i=1}^{n}(Ypred - Ymean)/n \quad (6)$$

where, Yobs and Ypred are the observed and predicted response variables, respectively, Ymean is the average of the Yobs values, and n denotes the sample number in the validation data set. RPIQ was defined as IQ/SEP, where SEP represents the standard error of prediction, and IQ denotes the interquartile distance of the validation set (IQ = Q3−

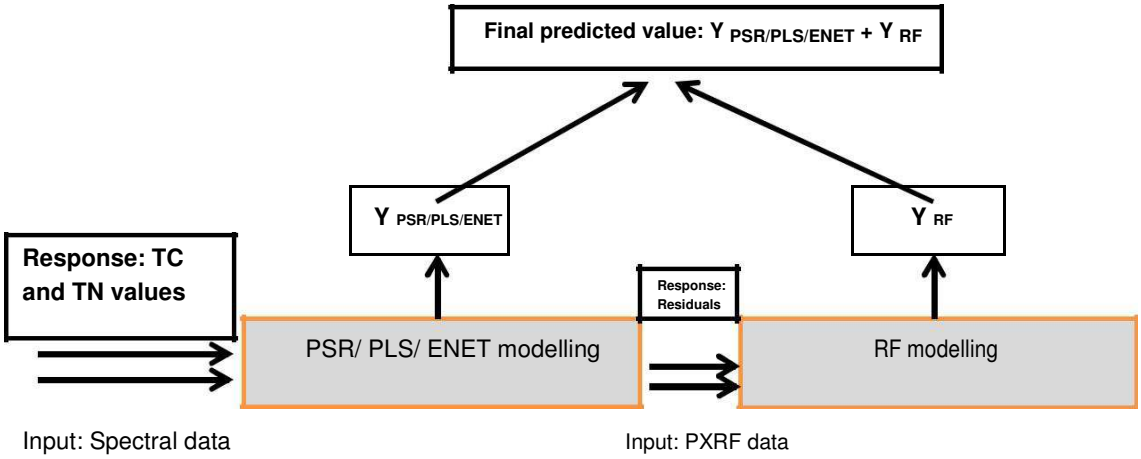


Fig. 1. Schematic diagram of fused PSR + RF, PLS + RF and ENET + RF prediction models used in the study.

Table 2
Summary statistics of soil properties.

Property	n	Mean	Std. dev	Median	Min	Max
Calibration dataset						
TC	96	39.53	8.74	41.64	0.06	51.3
TN	96	1.08	0.38	1.13	0.04	1.87
Validation dataset						
TC	40	37.9	8.33	38.07	11.42	51.1
TN	40	1.14	0.44	1.26	0.32	1.86
Whole dataset						
TC	136	39.05	8.63	40.95	0.06	51.3
TN	136	1.1	0.4	1.13	0.04	1.87

Q1). In this study, optimal model performance featured low RMSE values along with high RPD, R^2, and RPIQ.

3. Results and discussion

3.1. Soil properties and PCA

Statistical moments for TC and TN measured on the soil samples used in the regression models are listed in Table 2. Soil TC and TN showed negatively skewed distribution with mean concentrations of 39.05% and 1.10%, respectively for the whole dataset; 39.53% and 1.08%, respectively for the calibration set; and 37.90% and 1.14%, respectively for the validation set. Of the 13 elements obtained by the PXRF soil mode, only nine [Zn, S, K, Ca, Ti, Rb, Mn, Fe, Sr] were suitable for use in the multivariate predictive models with continuous data across all samples scanned. Within-region variability of TC and TN exhibited substantial variation. For instance, in Italy sample TC ranged from 28.06% to 45.94%. Showing similar variation, Lubbock samples showed TC ranging from 25.93% to 41.20%. Comparatively higher variability was observed for Houston samples (11.42–48.54%), while maximum TC variability was observed in samples from New Mexico region (0.06–51.30%). Lubbock samples showed TN ranging from 1.13–1.60%. Comparatively higher variability was observed for Italy (0.80–1.87%) and Houston samples (0.30–1.47%). Following a similar trend of TC, maximum variability in TN was observed from New Mexico soils (0.04–1.87%). Overall, variability in estimated TC and TN was due largely to the variability in the parent material, climate, C source, land use, and vegetation cover. However, the extent of variability appeared related with the range of the mean annual soil temperature of the considered sampling area, the highest in the New Mexico soils (2–13 °C), the lowest in the Italy soils (10–12 °C), intermediate in the other two subsets (16–20 °C in the Houston soils, and 13–17 °C in the Lubbock soils).

Table 3
Validation statistics of multivariate models based on Kennard-Stone splitting.

Soil property (%)	Approach	Model	R^2	PLS LF[a]	RMSE (%)	RPD	Bias	RPIQ
Total C	VisNIR DRS	PSR	0.80	–	3.46	2.40	0.15	3.02
		PLS	0.82	14	3.27	2.52	−0.15	3.23
		ENET	0.73	–	4.24	1.96	0.53	2.50
		RF	0.60	–	5.14	1.62	−0.03	2.06
	PXRF	PLS	0.53	5	5.59	1.49	0.89	1.89
		ENET	0.62	–	5.03	1.65	0.04	2.10
		RF	0.58	–	5.30	1.57	0.20	2.00
	VisNIR	PSR + RF	0.87	–	3.01	2.75	0.31	3.50
	DRS + PXRF	PLS + RF	0.89	14	2.94	2.84	0.22	3.60
		ENET + RF	0.79	–	3.73	2.23	0.56	2.84
	GRA	PSR.RF	0.59	–	5.27	1.58	−0.13	2.01
		PLS.RF	0.60	14	5.15	1.62	−0.13	2.06
		ENET.RF	0.62	–	5.05	1.65	−0.11	2.10
Total N	VisNIR DRS	PSR	0.81	–	0.178	2.46	0.03	3.80
		PLS	0.75	13	0.214	2.01	0.03	3.25
		ENET	0.74	–	0.221	2.00	0.05	3.14
		RF	0.61	–	0.271	1.62	−0.01	2.56
	PXRF	PLS	0.28	4	0.492	0.89	0.02	1.41
		ENET	0.32	–	0.356	1.23	0.01	1.95
		RF	0.57	–	0.285	1.54	0.01	2.43
	VisNIR	PSR + RF	0.85	–	0.160	2.62	0.03	4.12
	DRS + PXRF	PLS + RF	0.82	13	0.191	2.30	0.05	3.59
		ENET + RF	0.78	–	0.203	2.17	0.06	3.42
	GRA	PSR.RF	0.79	–	0.197	2.22	0.03	3.51
		PLS.RF	0.64	13	0.262	1.68	0.02	2.65
		ENET.RF	0.65	–	0.256	1.71	0.03	2.70

[a] PLS LF, partial least squares latent factor.

Levene's test yielded equality of variance of TC and TN values among the training and test sets ($p = 0.65$ and 0.55 for TC and TN, respective-ly). Moreover, t-test could not establish a significant difference between mean TC ($p = 0.53$) and TN ($p = 0.51$) for these two data sets. These results indicated that the validation samples based on the Kennard-Stone method can properly represent the studied population. The first two leading PCs constituted over 95% of the spectral variation (Fig. 2a). Although some overlapping among samples from four regions were discernible from the PC1 vs. PC2 plot, these regions had different ranges, shapes, and distributions in the spectral space, indicating variable SOM composition from different organic input sources (Fig. 2b). Moreover, the PCA plot indicating samples from three different organic horizons (OH, OLn and OLv) (Fig. 2c) indicated that OH horizon samples

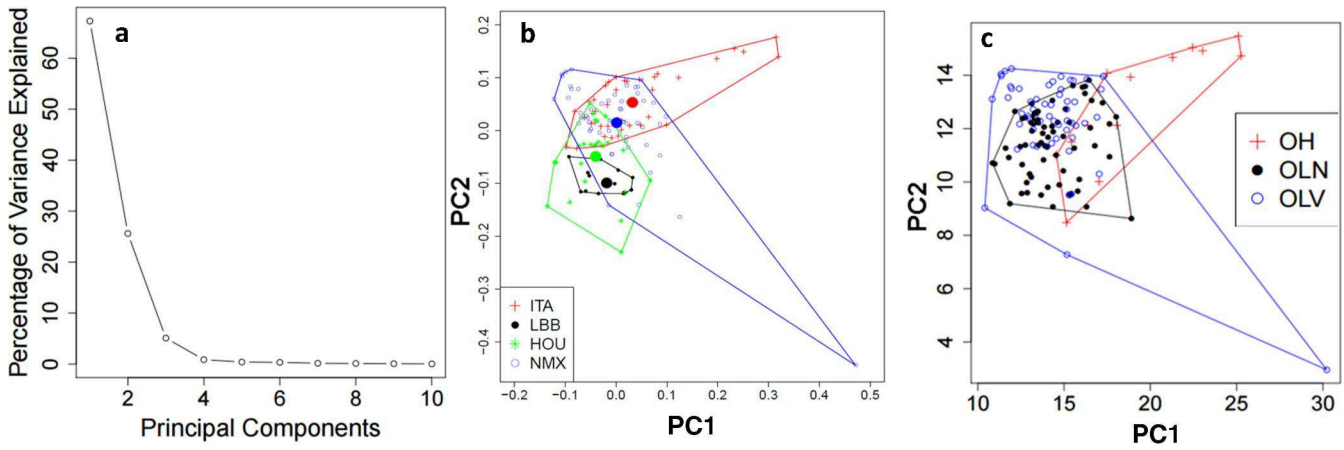


Fig. 2. Plots showing a) "Screeplot" of the first 10 principal components (PCs), b) pairwise PC plots of the first two components using VisNIR DRS first derivative spectra indicating four different geographical areas and c) pairwise PC plots of the first two components using VisNIR DRS first derivative spectra indicating three different organic horizons. Lines represent convex hulls and colored dots represent centroids of datasets from four different geographic areas. ITA, LBB, HOU and NMX represent samples from Italy, Lubbock, Houston and New Mexico regions, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

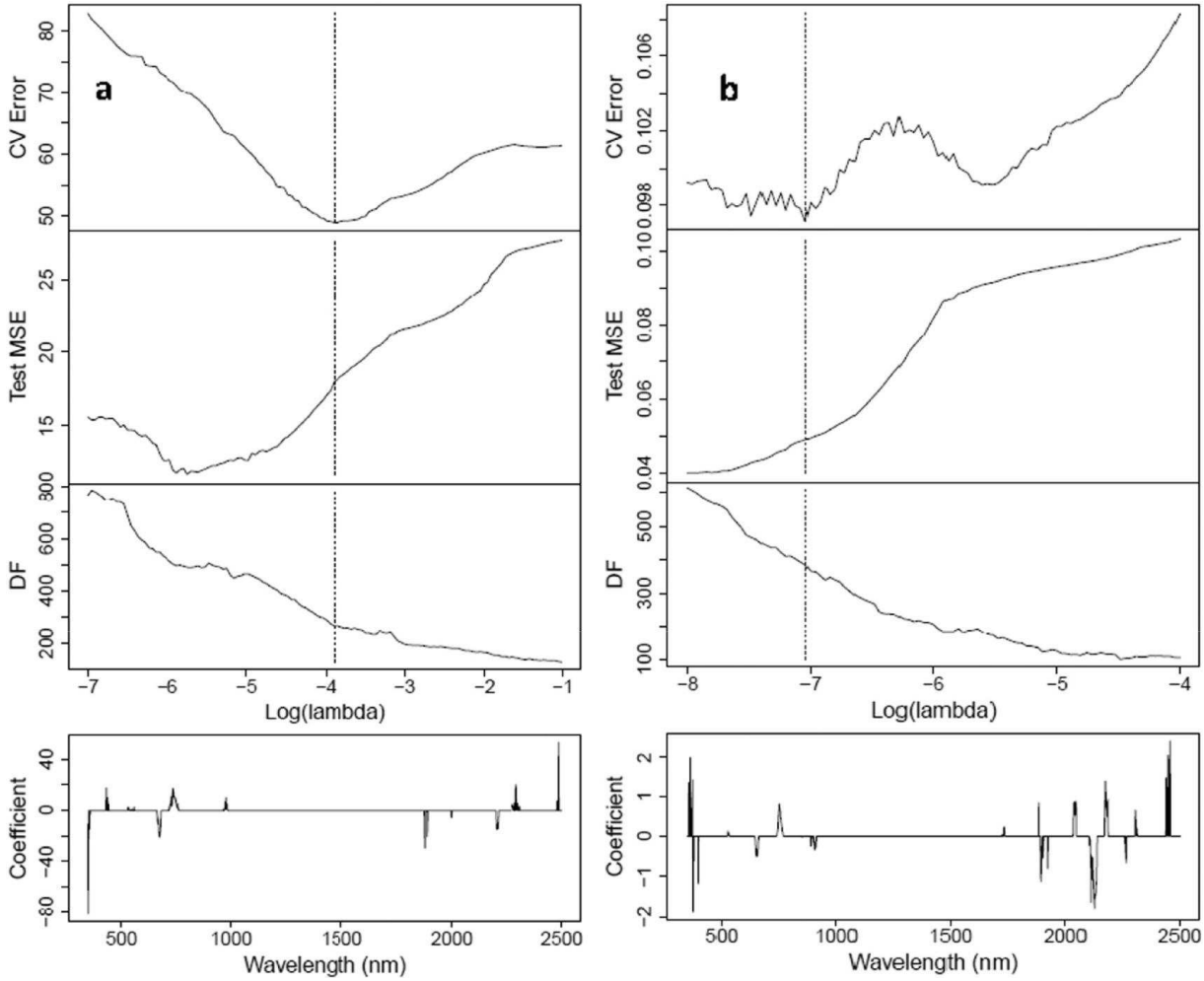


Fig. 3. Parsimony features of ENET for a) TC and b) TN. CV error, MSE, DF and λ represent cross-validation error, test error, the number of selected wavelengths and the parameter to tune the parsimony of the model, respectively.

were quite different from the samples from OLn and OLv horizons. The OH horizon samples tended to have larger values on the PC1 scores. Contrarywise, the samples from the OLn and OLv horizons were relatively close. Notably, the samples from the OLv horizon tended to have relatively larger PC2 values while there was an obvious outlying sample with the largest PC1 value and smallest PC2 value.

3.2. Validation results and model parsimony

In fused models, both TC and TN were estimated with good RPIQ values, better than using an individual instrument or GRA (Table 3), which has been noted before elsewhere (Wang et al., 2015). Fused (PLS + RF) and (PSR + RF) models yielded the highest RPDs for TC (2.84) and TN (2.62), respectively. While RPD values for both tested soil properties dropped in (ENET + RF) fused models, they still explained nearly 80% of the TC and TN variability. Overall, all three fused models of TC showed similar accuracies (Table 3), as reported for soil organic C in the review of Soriano-Disla et al. (2014) where the median R^2 of validations was calculated as 0.83 (33 studies).

Wavelength selection not only enhances the stability of the model but also makes the model more parsimonious. Although (PSR + RF) yielded the lowest RMSE for TN (0.160%), both PSR and RF are not parsimonious models since they do not select the subset of important variables. Although for RF it is possible to select different *mtry* values, which is the size of the candidate subset for each splitting, models with different *mtry* values still select all the variables. Despite controlling the smoothness of the neighboring coefficients through λ, PSR is also unable to select important wavelengths. Fig. 3 illustrates parsimony features of ENET for TC (Fig. 3a) and TN (Fig. 3b) where cross-validation error, test error (mean squared error or MSE), and the degrees of freedom (DF) (the number of selected wavelengths in the ENET model) were plotted against log of λ which is the parameter to tune the ENET parsimony. Note that the larger the λ, the more parsimonious the model. The vertical dash line shows the optimal λ that minimizes the cross-validation error. The bottom plot shows the ENET coefficient plot against the VisNIR wavelengths. For both soil properties, most of the coefficients were zero, indicating that they were not selected into the model. Additionally, based on the DF plots, it was evident that the optimal TC and TN models selected only ~300–350 out of 2151 wavelengths. Conversely, using a fused (PLS + RF) model on the reduced feature sets (14 and 13 latent factors for TC and TN, respectively) returned the best result for TC and slightly higher RMSE for TN (0.191%) than (PSR + RF) (0.160%) (Table 3). Based on these observations, we conclude that while considering both model precision and parsimony, (PLS + RF) emerged as the best model for both TC and TN.

For both TC and TN, while utilizing VisNIR-DRS or PXRF alone, RF and PLS models yielded the largest validation RMSE values, respectively, and were therefore the least precise (Table 3). In general, PXRF measurements were poorly correlated to both TC and TN, suggesting its limitation to make full geochemical assessment alone due to its inability to

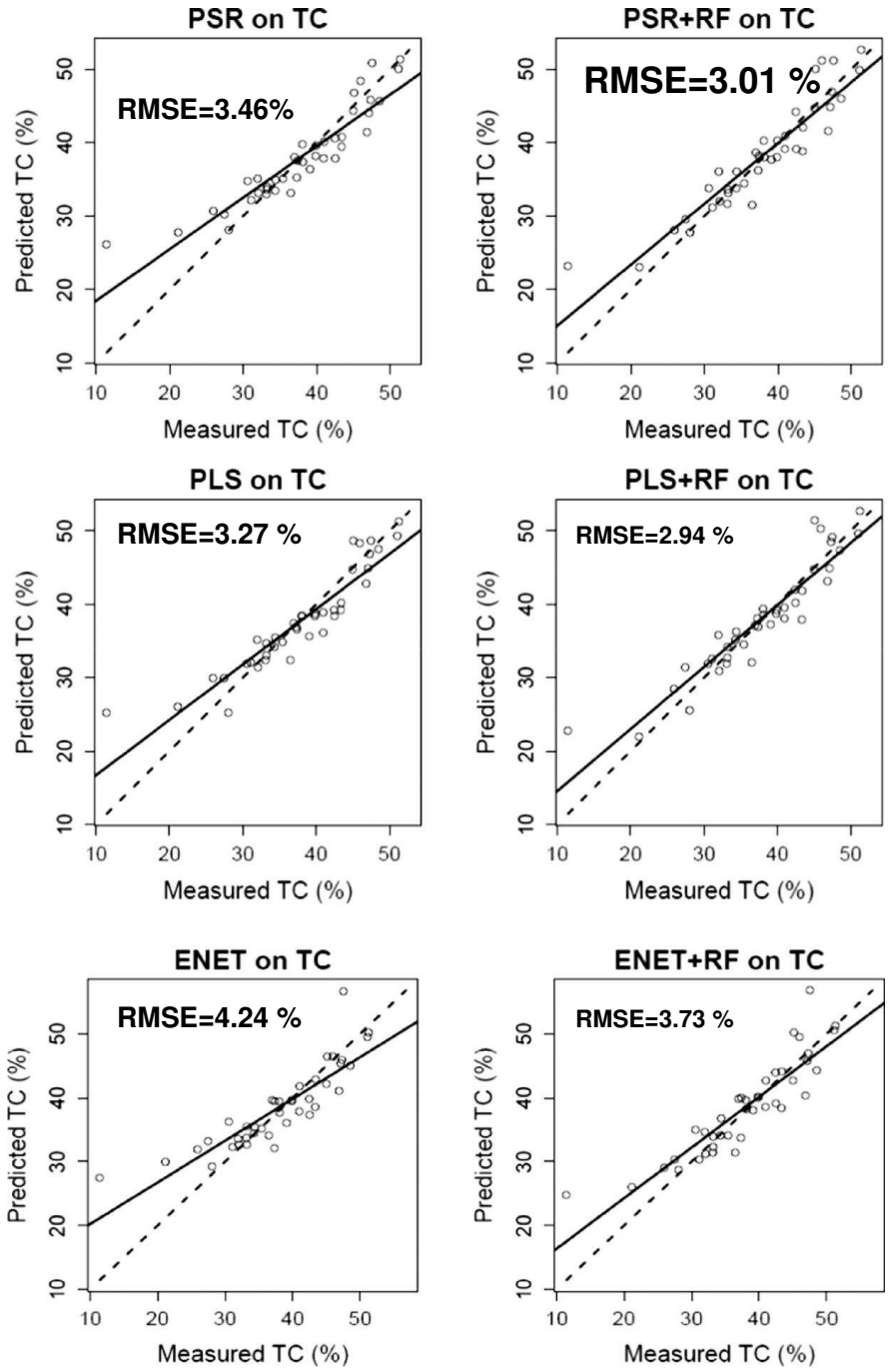


Fig. 4. Plots of observed vs. model predicted (VisNIR DRS only and fused) TC values using the validation set (with dotted 1:1 line).

handle low concentrations and light elements (Z ≤ 11) (Weindorf et al., 2008). Among the nine elements used in predictive models, only Al (1.48%), Ca (2.53%) and Si (4.83%) were present in high concentrations, while the other six elements were present at low levels (b1%). Thus, it seemed prudent to calibrate using PXRF spectra instead of elements if high accuracy is desired. While GRA substantially worsen the validation results for TC, (PSR + RF) model averaging for TN yielded close RMSE (0.197%) to those produced by fused models. The reasonably good performance of PSR in all three approaches (VisNIR only, fused, and GRA) can be attributed to the fact that in case of 'fat' data (large number of di-mensions and small sample size) PSR uses all samples and smooths constraints on the coefficient. Therefore, PSR often works well on signal regression problems which are usually strongly dimensional and have a relatively small sample size.

As coefficients of determinations of TN were rather similar to those of TC using VisNIR-DRS in isolation, we conclude that VisNIR-DRS sensed a grouping of soil components comprising organic functional groups that contain organic N fractions (Wang et al., 2015) (Table 3). Moreover, both TC and TN are spectrally active components or chromophores, which absorb incident energy at discrete energy levels and show broad and weak absorption features in the VisNIR region (Ben-Dor, 2011) Although intense VisNIR-DRS spectral bands cannot be di-rectly ascribed to metals or other components, Song et al. (2012) revealed that metals can interact with chromophores such as soil C.

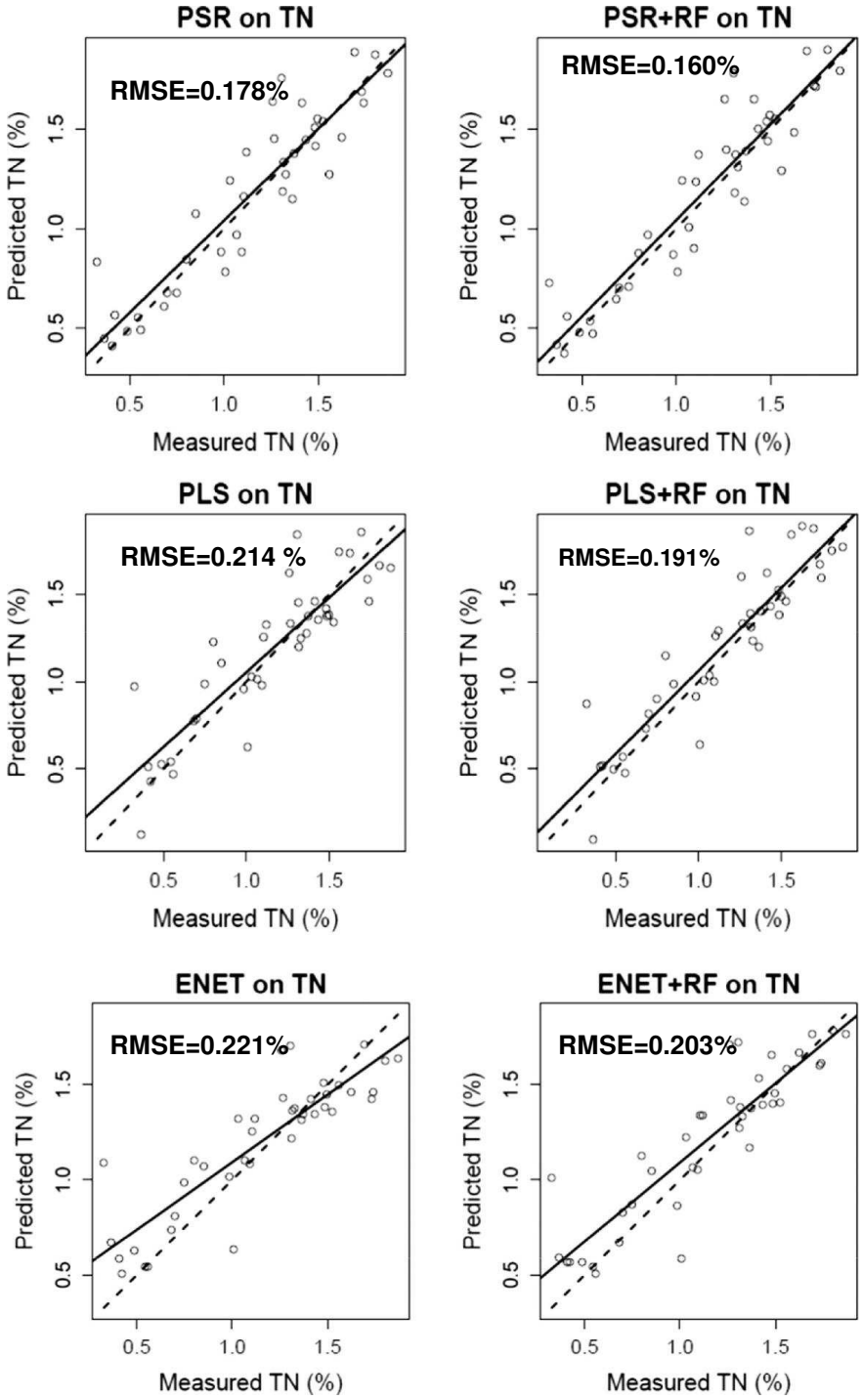


Fig. 5. Plots of observed vs. model predicted (VisNIR DRS only and fused) TN values using the validation set (with dotted 1:1 line).

However, S was negatively correlated with TC (ρ = –0.48); likely since S is very light with an atomic mass of 16 and was present in low concentrations (max = 0.43%).

Plots of observed vs. model predicted (VisNIR-DRS only and fused) TC and TN values are presented in Figs. 4 and 5, respectively. In general, all models showed overestimation at lower TC or TN values and underestimation at higher values. Several of these overestimations occurred because of the relative scarcity of observations with low values, which was expected since the samples came dominantly from organic horizons. Another possible explanation for TC and TN prediction errors is undecomposed organic matter and variable C sources, as noted before elsewhere (Henderson et al., 1992). Indeed, the VisNIR-DRS spectra for SOM have not been fully defined yet, because of the complexity or unclear definitions of these materials (Brown et al., 2006). Probably the TC and TN predictability could have been enhanced by using soil management or vegetation specific models (Morgan et al., 2009); nonetheless, exploring this notion was beyond the scope of this study.

3.3. Model fusion vs. model averaging

It was apparent that GRA model averaging by combining the VisNIR-DRS and PXRF predictions for consolidated use could not produce better accuracy than model fusion. In practice, “good” model averaging should contain the models that complement each other. In fused models, RF

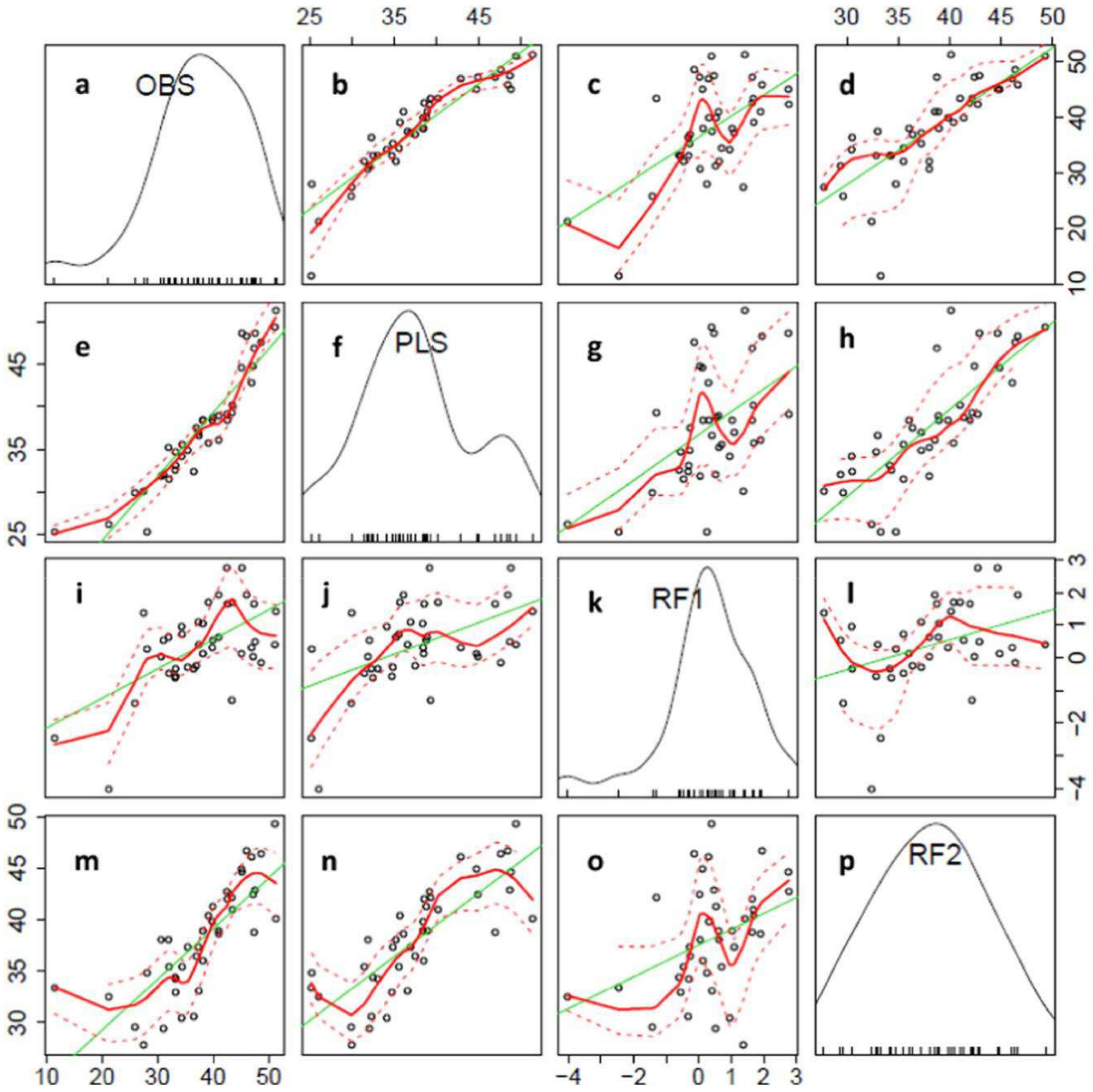


Fig. 6. Diagnostic scatter plot matrix showing density plots for four competitors: observed TC values (OBS), RF in (PLS + RF) fused model (RF1) and RF in GRA (RF2). Black circles represent validation samples. The green and red solid lines are the fitted linear regression line and the loess smoother fit, respectively. The red dash lines represent one standard error above and below the estimated function. For diagonal plots, the vertical axis shows the density function for its corresponding values. For example, panel a is the density plot of TC while the tickmarks at the bottom axis show the observed values in the data. In the off-diagonal plots, their axes are all in the % unit. For example, panel e shows the scatter plot of observed TC % (on the vertical axis) and predicted TC % values from PLS model (on the horizontal axis) using spectral data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was fitted based on the PLS/PSR/ENET residuals. The sequential fitting in PLS/PSR/ENET + RF allowed the RF model to complement the former. Conversely, combining PLS/PSR/ENET and RF in parallel fashion through GRA (namely, separately on VisNIR-DRS and PXRF) produced incompatibility. Succinctly, they perhaps made the same mistake on the same sample. To justify this postulation and clearly visualize the prediction improvement by using the fused models, Fig. 6 represents a scatterplot matrix produced in R using the spm function in the car library, taking the PLS + RF model as an example. The diagonal elements are the density plots for the four competitors [observed TC values (OBS), RF in (PLS + RF) fused model (RF1), and RF in GRA (RF2)]. For example, the upper left one is the density plot of TC while the tickmarks at the bottom axis show the observed values in the data. The off-diagonal elements are the pairwise scatter plots of four competitors, together with the best linear and nonlinear smoothers. For example, Fig. 6e shows the scatter plot of observed TC (on the vertical axis) and predicted TC values from PLS model (on the horizontal axis) using spectral data. The green and red solid lines are the fitted linear regression line and the loess smoother (a popular nonlinear smoother using local linear regression) fit, respectively. The red dash lines represent one standard error above and below the estimated function. It can be observed that PLS tended to underestimate the samples with TC values between 30 and 45% and overestimate the samples with TC values <20% through the bending shape of the red loess smoother (Fig. 6e). Interestingly, while RF1 “corrected” the PLS model by lifting the underestimated area (30–45% TC) and lowering the overestimated region (<20% TC) (Fig. 6i), this was not totally

Table 4
Relative improvement of % RMSE and RPIQ in fused models.

Soil property (%)	Approach	Model	RMSE (%)	RPIQ	Comparing approach	Model	% improvement in RMSE	% improvement in RPIQ
Total C	VisNIR DRS	PSR	3.46	3.02	VisNIR DRS + PXRF	PLS + RF	15.02	19.20
		PLS	3.27	3.23			10.09	11.45
		ENET	4.24	2.50			30.66	44.00
		RF	5.14	2.06			42.80	74.75
	PXRF	PLS	5.59	1.89			47.40	90.47
		ENET	5.03	2.10			41.55	71.42
		RF	5.30	2.00			44.50	80.00
	GRA	PSR.RF	5.27	2.01			44.21	79.10
		PLS.RF	5.15	2.06			42.90	74.75
		ENET.RF	5.05	2.10			41.78	71.42
Total N	VisNIR DRS	PSR	0.178	3.80	VisNIR DRS + PXRF	PSR + RF	10.11	08.42
		PLS	0.214	3.25			25.23	26.76
		ENET	0.221	3.14			27.60	31.21
		RF	0.271	2.56			40.95	60.93
	PXRF	PLS	0.492	1.41			67.47	192.19
		ENET	0.356	1.95			55.05	111.28
		RF	0.285	2.43			43.85	69.54
	GRA	PSR.RF	0.197	3.51			18.78	17.37
		PLS.RF	0.262	2.65			38.93	55.47
		ENET.RF	0.256	2.70			37.50	52.59

unexpected owing to the non-linear and contingent relationships among VisNIR-DRS reflectance and soil constituents (Brown et al., 2006). However, RF2 made the same "mistake" as PLS by underestimating and overestimating in the same regions (Fig. 6d). These trends clearly explained why sequentially fitting (PLS + RF) in fused model improves prediction accuracy relative to parallel fitting of PLS and RF through GRA. Note that, the same argument is applicable to (PSR + RF) or (ENET + RF) in predicting TC and TN. Model averaging even could not outperform VisNIR-DRS only model performance (Table 3). Thus, the postulations of Abbott (2014), who reported that model averaging nearly always improves model predictive accuracy and rarely predicts worse than single models, may not be generalized.

3.4. Relative improvement of soil property predictions after model fusion

Table 4 demonstrates that fused models had relative improvements on the prediction accuracy of TC and TN from GRA or VisNIR-DRS and PXRF used in isolation. Notably, RMSE improvements of 10–47% for TC and 10–67% for TN were achieved by the PLS + RF and PSR + RF models, respectively, demonstrating the potential of the synergistic use of VisNIR-DRS and PXRF for the estimation of soil attributes. On average, the PLS + RF model for TC and the PSR + RF model for TN produced almost a one fourth reduction in RMSE from using VisNIR-DRS in isolation. While the PLS + RF fused model increased RPIQ values ranging between 11 and 97% for TC, the PSR + RF model for TN yielded three times higher RPIQ than using PXRF in isolation. Also, the PSR + RF approach for TC produced substantively better results (R^2 0.87; RMSE 3.01%; RPD 2.75) than those obtained by McWhirt et al. (2012) (R^2 0.82; RMSE 10.1%; RPD 1.72) who used VisNIR-DRS in isolation when evaluating the organic fraction of composted products.

3.5. Whole geographical area and organic horizon holdout validations

Holding out a whole geographical area or organic horizon reduced prediction accuracies in all cases where the soils of the geographical area or horizon held out were not represented by another area or horizon, creating extrapolation (Tables 5 and 6). Validation with an individual geographical area or horizon exhibited increases in RMSEs and a reduction in RPDs relative to the best performing models (PLS + RF and PSR + RF for TC and TN, respectively) using all 136 samples. As expected, when principal component analysis was implemented on the VisNIR spectrum only, it clearly demonstrated different spectral behaviors of the four geographical areas (Fig. 2b). Further, while executing Principal Component Regression (PCR, data not shown) to calculate the actual number of PCs required to represent VisNIR spectral variability, results indicated the need for at least 12 PCs in PCR based on full cross validation. In other words, although some overlap among the four areas was apparent in the PC1 vs. PC2 plot, that only reflected the first two PCs. Summarily, it can be concluded that since the four geographical areas had different VisNIR spectra, using all samples in a single prediction model is preferred over the area-holdout scheme since the latter involves extrapolation in predicting samples from different areas.

Moreover, based on the whole organic horizon-holdout results, all models predicted poorly on holding out OH and OLn samples. For OH, the main reason of poor model performance was that OH samples were quite different from OLn and OLv samples because of variable rates of decomposition. Hence the fitted models using OLn and OLv

Table 5
Prediction accuracies of total C and total N using whole geographical area holdout valida-tion. Models were created using PLS + RF model (for total C) and PSR + RF model (for total N) with three geographical areas and then validated using the fourth area. Eight separate models were calibrated and validated.

Validation location	Parameter	RMSE (%)	RPD	Bias	RPIQ
Italy	Total C	5.26	0.92	1.19	1.07
Lubbock		2.84	1.57	−2.04	2.73
Houston		5.16	1.92	−0.41	1.26
New Mexico		12.55	0.80	−0.52	0.70
Italy	Total N	0.28	0.99	−0.11	1.30
Lubbock		0.16	0.72	−0.09	0.93
Houston		0.24	1.15	0.13	1.26
New Mexico		0.99	0.40	0.42	0.58

Table 6
Prediction accuracies of total C and total N using whole-organic horizon holdout validation. Models were created using PLS + RF (for total C) and PSR + RF (for total N) with two organic horizons and then validated using the third organic horizon. Six separate models were calibrated and validated.

Validation location	Parameter	RMSE (%)	RPD	Bias	RPIQ
OH	Total C	9.22	0.77	5.90	1.14
OLn		10.32	0.90	3.42	1.05
OLv		7.17	1.01	−1.83	0.79
OH	Total N	0.47	0.82	0.23	1.16
OLn		1.19	0.31	0.16	0.40
OLv		0.34	1.25	0.05	2.00

samples could not predict OH samples well. Conversely, since the OLn horizon represented most of the samples (i.e. 64 out of 136 samples), the sample size for fitting the model using the OLv and OH was the smallest. In addition, the OH samples in the training data made the fitted model poor in predicting OLn samples in the validation set.

3.6. Some practical concerns

The fused PSR + RF model had some limitations too. Initially, PSR was applied on VisNIR-DRS spectra with the assumption that the underlying PSR coefficient curve is smooth. Secondly, RF was used to fit the residuals of the PSR model on the PXRF elemental data assuming that both VisNIR-DRS and PXRF data contain some information on the response variable. In particular, PXRF contains some extra information about TC and TN that were not explained (or covered) by the VisNIR-DRS spectra. We have examined the feasibility of the approach in this research and have provided a preliminary contribution to the issue of validation, however, further intensive research is recommended to confirm our results. Nonetheless, the combined use of PXRF and VisNIR data shows strong potential for the accurate assessment of soil organic horizons. The spectral and elemental data produced by proximal sensor analysis has previously been shown to be useful in the prediction of a wide num-ber of physicochemical properties in mineral soils. With regard to TC and TN content of organic soil horizons, similar impressive predictability from proximal sensor data was observed.

4. Conclusions

Summarily, this preliminary study of 136 organic laden soil samples from non-saturated uplands of Italy and the United States was evaluated by standard laboratory analysis as well as proximal sensor (PXRF + VisNIR DRS) scans. Our results show that:

(i) Combining model outcomes using model fusion improved TC and TN prediction accuracies relative to using an individual instrument (PXRF or VisNIR DRS) or model averaging. Overall, the relative improvement in % RMSE ranged from 10 to 47% and 10–67% for TC and TN, respectively.

(ii) Considering both model precision and parsimony, PLS + RF emerged as the best model for predicting both TC and TN in organic laden soil samples. Conversely, while using all spectral variables, PSR + RF yielded the best model results (lowest RMSE and highest RPIQ) for TN.

(iii) Specifically, GRA model averaging by combining the VisNIR-DRS and PXRF predictions could not produce better accuracy than model fusion or individual instrument use, possibly due to model incompatibility while fitting them in parallel fashion.

(iv) The poor predictability with unreliable RPIQs while using PXRF in isolation underlines the need for using PXRF spectra instead of elemental data.

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Effect of beech (*Fagus sylvatica* L.) rhizosphere on phosphorous availability in soils at different altitudes (Central Italy)

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abstract

Phosphorus (P) is an important nutrient for plant growth but its availability in soil is limited. Although plants are able to respond to the P shortage, climatic factors might modify the soil-plant-microorganisms system and reduce P availability. In this study we evaluated the rhizosphere effect of beech (Fagus sylvatica L.) in forest soils of Apennines mountains (central Italy) at two altitudes (800 and 1000 m) and along 1° of latitudinal gradient, using latitude and altitude as proxies for temperature change. Specifically, we tested if 1) soil organic C, total N, and organic and available P decrease with increasing latitude and altitude, and 2) the rhizosphere effect on P availability becomes more pronounced when potential nutrient limitations are more severe, as it happens with increasing latitude and altitude. The results showed that the small latitudinal gradient has no effect on soil properties. Conversely, significant changes occurred between 800 and 1000 m above sea level, as the soils at higher altitude showed greater total organic C (TOC) content, organic and available P contents, and alkaline monophosphatases activity than the soils at lower altitude. Further, at the higher altitude, a marked rhizosphere effect was detected, as indicated by greater concentration of TOC, water extractable organic C, and available P, and its fulfillment was mainly attributed to the release of labile organics through rhizodeposition. The availability of easy degradable compounds in the rhizosphere should foster the mineralization of the organic matter with a consequent increase of available P. Hence, we speculate that at high altitude the energy supplied by the plants through rhizodeposition to the rhizosphere heterotrophic microbial community is key for fuelling the rhizospheric processes and, in particular, P cycling.

1. Introduction

Phosphorus (P) is one of the most critical nutrients for the growth of plants and microorganisms, and is present in soil both in inorganic and organic forms. The inorganic P includes primary minerals (e.g., apatites, strengite) and secondary minerals such as calcium-, iron-, and aluminum-phosphates (Shen et al., 2011), while the organic P is mainly made up of phospholipids, orthophosphate monoesters, nucleic acids, teichoic acids and phosphonates (Rubæk et al., 1999; Kruse et al., 2015). The content and distribution of these different P forms do not have a constant trend along the soil profile (Makarov et al., 2004; Chiu et al., 2005: Backnäs et al., 2012). In grassland and beech forest soils located on north-facing slope of southern Swiss Alps, Beck and Elsenbeer (1999) found that the variation with depth of the organic P concentration mainly depends on type and age of vegetation, and soil characteristics. Then, Chiu et al. (2005) studied grassland and coniferous forest soils and found that the inorganic orthophosphate concentration decreased, but orthophosphate monoesters increased with increasing soil depth. The generally low availability of P in soil is due to the scarce solubility of P-bearing compounds, both for inorganic and organic forms (Hinsinger, 2001). For example, in the soil solution P exists as ortho-phosphate anions and dissolved forms of organic P, with concentrations that range from 0.01 to 3.0 mg l^{-1} (Frossard et al., 2000). However, plants are able to respond to P deficiency by root-exudation of organic acids that increase P availability in the soil close to the roots (Ström et al., 2002; Oburger et al., 2009; Zhao et al., 2010). Furthermore, the simple organic compounds comprising the plant exudates enhance the activity of the microorganisms that, in turn, favours P availability by i) soil acidification due to the CO_2 produced through respiration, and ii) releasing organic acids and phosphatase enzymes (Marschner et al., 2011). A great part of the complex interactions among P-bearing compounds, plant and microorganisms occurs in the small soil volume between fine roots and earthy material, the rhizosphere, where most of the chemical, biochemical and biological reactions take place (e.g., Hinsinger et al., 2003; Richter et al., 2007; Lambers et al., 2009). Because of its high sensitivity to the environmental conditions

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(Turpault et al., 2007), the biogeochemical processes occurring in the rhizosphere can be strongly affected by climate. Indeed, climate (temperature, precipitation amount and pattern) is one of the key soil forming factors that inherently controls the soil profile development (Darwish and Zurayk, 1997; Fernández-Sanjurjo et al., 2003; Riebe et al., 2004), soil microbial activity (Qiu et al., 2005; Devi and Yadava, 2006), soil organic matter dynamics (Jobbagy and Jackson, 2000; Brevik, 2013), and macro- and micro-nutrients cycles (Butler et al., 2012; Vincent et al., 2014; Zhang et al., 2014). For example, in a phytotron experiment, Kumar et al. (2011) found that higher temperatures increased the amount of available P, but decreased organic P in the rhizosphere of wheat. Instead, as far as we are aware, in vivo studies on the P dynamics in the rhizosphere of forest species at different temperature have never been achieved, although they could help better understanding the resilience of forest ecosystems with respect to global (and more regionally localized) warming. The objective of this study was to provide novel field and laboratory information on this argument. To assess the influence of temperature on the rhizospheric P pool, we selected one of the main diffused forest species in Europe, i.e. the European beech (Fagus sylvatica L.). By using latitude and altitude as proxies for temperature change (Vincent et al., 2014), we contrasted the associated changes in European beech rhizosphere and bulk soil collected from the different horizons of forest soils at two altitudes (800 and 1000 m) on three mountains located within 1° of latitudinal gradient in central Italy. Specifically, we tested the hypotheses that: 1) soil organic C, total N, and organic and available P decrease with increasing latitude and altitude, and 2) the rhizosphere effect on P availability becomes more pronounced when potential nutrient limitations are more severe, as it happens with increasing latitude and altitude. The above two hypotheses were tested on rhizosphere and bulk soil from the three study areas through measuring of organic C, total N, organic and available P, and phosphatase activities; additionally, ^{31}P NMR analyses were performed to assess the different forms of soil P.

2. Materials and methods

2.1. Study sites

As study areas, three calcareous massifs were selected on the Apennines chain (central Italy): Mount Terminillo (42°28′ N, 12°59′ E), Mount San Vicino (43°19′ N, 13°03′ E), and Mount Acuto (43°28′ N, 12°41′ E) (Figure S1 of the Supplementary Materials). For each area, two European beech (Fagus sylvatica L.) forests were chosen on the north-facing slopes at about 800 and 1000 m above sea level (a.s.l.). A description of the environmental conditions of each site is reported in Table 1. Here it suffices to summarize that the three areas have a similar mean annual air temperature (MAAT) that varies from about 10 °C at 800 m, to about 9 °C at 1000 m a.s.l. Following the latitudinal transect, coldest and warmest months showed a contrasting trend as, going north, the mean of the coldest month (January) decreased of 1.6– 2.0 °C, while that of the warmest month (July) increased of 1.6–1.7 °C. In all the areas, the mean temperature for both coldest and warmest months is lower in the soils at 1000 m than in those at 800 m a.s.l. of 0.6–1.0 and 1.0–1.1 °C, respectively. The mean annual precipitation, similar at both altitudes, do not follow a latitudinal transect, and is the highest at Mount Acuto, the lowest at Mount San Vicino.

All the forests were coppices in conversion, with the conversion that started from about 20 to about 40 years ago. As indicated by the diameter at breast height (Table 1), the most recent conversions occurred at Mount Terminillo and Mount San Vicino, at 800 and 1000 m a.s.l., respectively; the oldest conversions started at Mount Terminillo for the coppice at 1000 m a.s.l., and at Mount Acuto for the woods at both altitudes. At all sites beech was the dominant tree, with dominances ranging from 80 to 100%. While the soil cover due to litter was always complete, the coverage due to understory ranged from 5 to 50%, with scarce to null signs of erosion.

Table 1
General features of the study sites at three latitudes (Mt. Terminillo, Mt. San Vicino, and Mt. Acuto). Central Apennines, Italy. All the selected forests were in conversion from coppices to high forest.

	Mt. Terminillo (max height 2217 m)		Mt. San Vicino (max height 1480 m)		Mt. Acuto (max height 1668 m)	
	800 m	1000 m	800 m	1000 m	800 m	1000 m
Exposure	N	N	N	N	NNE	NNE
Mean slope	40°	40°	35°	45°	40°	25°
MAAT	10.0 °C (1956–2014 series)	9.1 °C (1956–2014 series)	10.0 °C (1950–2000 series)	9.0 °C (1950–2000 series)	10.0 °C (1950–2000 series)	9.0 °C (1950–2000 series)
Coldest month	January (1.6 °C)	January (1.0 °C)	January (1.0 °C)	January (0.0 °C)	January (0.0 °C)	January (−1.0 °C)
Warmest month	July (19.4 °C)	July (18.3 °C)	July (21.0 °C)	July (20.0 °C)	July (21.0 °C)	July (20.0 °C)
MAP	1248 mm (1956–2014 series)		825 mm (1950–2000 series)		1430 mm (1950–2000 series)	
Parent material	Grey limestone with small layers of flintstone (Lower Jurassic, Toarcian-Sinemurian)		Grey limestone with layers of marl and flintstone (Lower Jurassic, Pliensbachian-Sinemurian)		White limestone with layers of flintstone (Lower Cretaceous, Aptian)	
Dominant trees	*Fagus sylvatica* (95%), *Acer* spp., *Laburnum anagyroides*	*Fagus sylvatica* (99%), *Acer* spp.	*Fagus sylvatica* (90%), *Quercus cerris*, *Castanea sativa*	*Fagus sylvatica* (80%), *Sorbus aria*, *Quercus cerris*	*Fagus sylvatica* (95%), *Carpinus betulus*	*Fagus sylvatica* (100%)
Understory	25% of soil cover made of *Edera elix*, *Clematis vitalba*, *Daphne laureola*, *Lamium* spp., *Viola* spp., Gramineae, mosses, *Juniperus communis*, *Fagus* seedlings	5% of soil cover made of *Fagus* seedlings	15% of soil cover made of *Rubus ulmifolium*, *Pteridium aquilinum*, *Daphne laureola*, mosses, *Cyclamen repandum*, *Clematis vitalba*	5% of soil cover made of Graminae, *Crategus oxyacantha*, *Rosa canina*, *Edera elix*, *Cyclamen repandum*, mosses	30% of soil cover made of *Edera elix*, Gramineae, *Cyclamen repandum*, *Ruscus aculeatus*, *Daphne laureola*, *Allium ursinum*, *Pteridium* spp., *Viola* spp., mosses	50% of soil cover made of *Daphne laureola*, *Edera elix*, *Viola* spp., *Allium ursinum*, *Lamium* spp., *Stellaria* spp.
DBH of the two selected beeches	22.3 cm	41.4 cm	26.4 cm	21.8 cm	40.7 cm	38.5 cm
	22.3 cm	37.9 cm	22.4 cm	23.5 cm	41.2 cm	39.3 cm
Soil classification	Typic Hapludoll	Lithic Haprendoll	Typic Hapludoll	Lithic Haprendoll	Typic Humudept	Inceptic Haprendoll
	Lithic Haprendoll	Lithic Haprendoll	Typic Humudept	Inceptic Haprendoll	Typic Humudept	Inceptic Haprendoll

MAAT: mean annual air temperature; MAP: mean annual precipitation; DBH: diameter at breast height.

All the soils had developed from limestone rocks with small flintstone layers.

2.2. Soil sampling

During the winter 2014, at each altitudinal site two profiles were dug within a plot of about 100 m^2, for a total of 12 profiles (3 latitudes × 2 altitudes × 2 profiles). The rational of the winter sampling was that, in this season, root respiration and exudation, and root-associated microorganism activity are at their lowest intensity (Epron et al., 2001; Buée et al., 2005; Meinen et al., 2009; Ruehr and Buchmann, 2010; Calvaruso et al., 2014); hence, more reliable and stable information on the rhizosphere status can be obtained in winter rather than in more dynamic seasons like spring or summer. Each profile was opened at 50–60 cm from the stem of the biggest beeches found in the selected site. Approximately, the age of the trees ranged from about 40 years at Mount Terminillo (800 m a.s.l.) to about 60–65 years at Mount Acuto. However, the age of the tree is of secondary importance as, for the protocol adopted to obtaining the rhizosphere samples (see below), we considered only the fine roots, which activity is little dependent on the age of adult plants as they are renewed every few year (Trumbore and Gaudinki, 2003; Agnelli et al., 2014).

The profiles were dug till the parent rock, and the soils were morphologically described according to Schoeneberger et al. (2012). As a whole, the soils at 800 m a.s.l. showed a solum made of the following sequence of horizons, with respective mean thicknesses (standard devia-tions in parentheses): O = 7.2 cm (2.4), A = 7.0 cm (3.2), AB = 7.0 cm (1.5), Bw1 = 13.2 cm (3.7), Bw2 = 12.8 cm (4.7), Bw3 = 26.5 cm (8.9), Bw4 = 18.3 cm (4.2). The soils at 1000 m a.s.l. showed the following solum: O = 10.2 cm (6.2), A = 10.3 cm (3.2), AB = 14.2 cm (7.7), Bw1 = 19.8 cm (9.4), Bw2 = 10.0 cm (4.7). The mean thickness (ex-cluding the O horizons) of the solum was 68.8 cm (36.3) at 800 m, and 49.2 cm (19.9) at 1000 m. The underneath C horizons are not part of the solum and were excluded from sampling. The litter was made by O horizons that were typical of the amphimus type of humus (Baize and Girard, 2008), which are present in soils with well-developed O horizons rich of pedofauna, and resting on A horizons with well-developed crumb structure.

Roughly, in the soils at 800 m the fine earth content ranged from 85% in the A horizon to 50% in the Bw3 and Bw4 horizons, while in those at 1000 m it went from 80% in the A horizon to 35–40% in the Bw1 and Bw2 horizons. All the soils had a mesic soil temperature regime, and an udic soil moisture regime, and were classified as Mollisols or Inceptisols (Table 1) according to the Soil Survey Staff (2014).

For each profile, a large amount of sample (at least 3 kg) from each mineral horizon forming the solum was collected and stored in a porta-ble refrigerator for the transport to the laboratory.

2.3. Sample preparation

Within one week from the sampling, the beech rhizosphere of each sample was isolated by picking up the roots together with the adhering soil (Cocco et al., 2013; Massaccesi et al., 2015). The roots with a diam-eter larger than 2 mm were discarded. After a light shaking to detach the weakly adhering soil particles, which were then added to the bulk soil (i.e., the soil not strictly adhering to the roots), the remaining soil mate-rial firmly adhering to the fine roots was considered as rhizosphere and was recovered by further shaking and gentle brushing. An aliquot of the field moist rhizosphere and bulk of each horizon was stored at 4 °C for measuring the phosphatase activities, while the remaining material was air-dried and sieved through a 2 mm mesh to be used for the chem-ical and spectroscopic analyses.

2.4. Chemical analysis

The soil pH was determined potentiometrically in water (pH_{H2O}) and in 1 M KCl solution (pH_{KCl}) (solid:liquid ratio of 1:2.5) after 30 min of stirring by a combined glass-calomel electrode. Total organic C content (TOC) was estimated by K-dichromate digestion, heating the suspension at 180 °C for 30 min (Nelson and Sommers, 1996), and total N content was determined by a Carlo Erba EA1110 dry combustion an-alyzer (Carlo Erba Instruments, Italy). To measure the water extractable organic C (WEOC), 1 g of sample was placed into a plastic container, submerged with distilled water (solid:liquid ratio 1:10) and shaken overnight with an orbital shaker (140 rpm). The mixture was left to rest for a while, centrifuged at 1400g for 10 min, and then filtered through Whatman 42 filter paper. The resulting solution was analyzed with a TOC-500A (Shimatzu, Japan) analyzer after the addition of a few drops of concentrated H_3PO_4 to eliminate carbonates. The total P was determined by the ignition method, and the organic P was calculat-ed by difference between the total and inorganic P content (Kuo, 1996). Available P was estimated according to Olsen et al. (1954).

Acid and alkaline mono-phosphatase activities were determined according to Tabatabai (1994). Briefly, in a 50-ml flask, 1 g of sample was added with 0.2 ml of toluene, 4 ml of modified universal buffer (at pH 6.5 and pH 11 for the acid and alkaline phosphatases, respectively), and 1 ml of 0.025 M p-nitrophenyl phosphate (p-NPP) solution. The mixture was incubated at 37 °C for 1 h so to induce the transformation of p-NPP into p-nitrophenol (p-NP) via phosphatase. After incubation, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added to the samples, mixed for few seconds, and then filtered through a Whatman 42 filter paper. The color intensity of the filtrate was measured against a blank at 420 nm by a Lambda EZ 150 UV/VIS Spectrometer (Perkin Elmer, USA). The results are expressed as μg of p-NP produced by 1 g of soil per h of incubation (μg p-NP g^{-1} h^{-1}).

For the ^{31}P NMR measurements, which were run only on the A horizons, the extracts were obtained by shaking 5 g of sample with 100 ml of a solution containing 0.25 M NaOH and 0.05 M EDTA for 16 h at room temperature, in the dark. The mixture was centrifuged at 10,000g for 30 min and the liquid phase, once separated from the precipitated, was freeze-dried. The freeze-dried NaOH-EDTA extracts were re-dissolved in 0.1 ml of 10 M NaOH solution and 0.5 ml of D_2O, and trans-ferred to a 5-mm NMR tube for the analysis. The ^{31}P NMR spectra were obtained using an Arance 600 MHz NMR spectrometer (Bruker, USA) operating at 243 MHz, with an acquisition time of 0.673 s, and a delay time of 0.5 s. For each sample 24,576 scans were run. Peak assignments were according to Turner et al. (2003), and the intensities of signals were determined by integration.

The accuracy of the run soil analyses follows: pH in water, 0.14; pH in KCl, 0.11; TOC, 2.05 g kg^{-1}; total N, 0.19 g kg^{-1}; WEOC, 0.024 g kg^{-1}; total P, 35 mg kg^{-1}; organic P, 24 mg kg^{-1}; available P, 2.5 mg kg^{-1}; acid and alkaline mono-phosphatase activity, 5 μg p-NP g^{-1} h^{-1}.

2.5. Statistical analyses

To test the effect of each variable (latitude, altitude, soil horizons, and soil fractions) on the soil properties we performed canonical redundancy analyses (RDA). The RDA model was tested for significance using 999 random permutations. The variations of soil properties as a function of latitude, altitude, soil horizons, and soil fractions were assessed by a Principal Component Analysis (PCA). For each property, all the data were standardized prior the RDA and PCA by subtracting the mean and dividing by the standard deviation.

The RDA indicated a lack of significant effect for latitude (ANOVA, F = 2.39, P = 0.057), whereas the effects of altitude (ANOVA, F = 15.84, P = 0.001), soil horizons (ANOVA, F = 4.13, P = 0.001) and soil fractions (ANOVA, F = 62.86, P = 0.001) on the soil properties were significant. Because of this, only these three latter significant

variables were considered in further detail in our study, and the three latitudinal areas were therefore considered as replicates. Consequently, the analytical results obtained from the two samples collected for each horizon at each latitude and altitude were averaged, and these averages used as replicates so to have n = 3. The data were checked for the normality of the distribution and the homogeneity of the variances by Shapiro-Wilk and Levene tests, respectively, and, if necessary, transformed by the Box and Cox (1964) procedure. To assess significant differences among altitudes, horizons and soil fractions (rhizosphere and bulk soil), three-way ANOVA was performed, and the comparison of means was assessed by Fisher's LSD post-hoc test ($P < 0.05$). Box plot diagrams were used to show the obtained data. The line inside each box represents the median. The bottom and top of the box are the first and third quartiles, while the upper and lower whiskers indicate the minimum and maximum values, respectively; the + sign within each box plot indicates the average.

The statistical analyses were performed using R software (R Core Team, 2014).

3. Results

3.1. PCA

The PCA scoring plot (Figure 1) showed variations of the soil properties between rhizosphere and bulk and between the soils at 800 and 1000 m a.s.l., and identified the axes 1 and 2 that explained about 53% and 24% of the variation, respectively. All the soil parameters showed positive correlation with PC1 (Figure S2a of the Supplementary Materials), with TOC, alkaline and acid phosphatase activities, total N, and available and organic P contributing for N83% to the variability (Table 2). While the pH_{H2O} and pH_{KCl} only slightly affected PC1, they explained 57% of the variability along PC2 axis. The scoring plot (Figure 1) showed that the soil properties were affected mainly by altitude and soil fractions and to a lesser extent by soil horizons. Moreover, the PCA indicated that the differences between the rhizosphere and bulk were somewhat greater at 1000 m than at 800 m a.s.l.

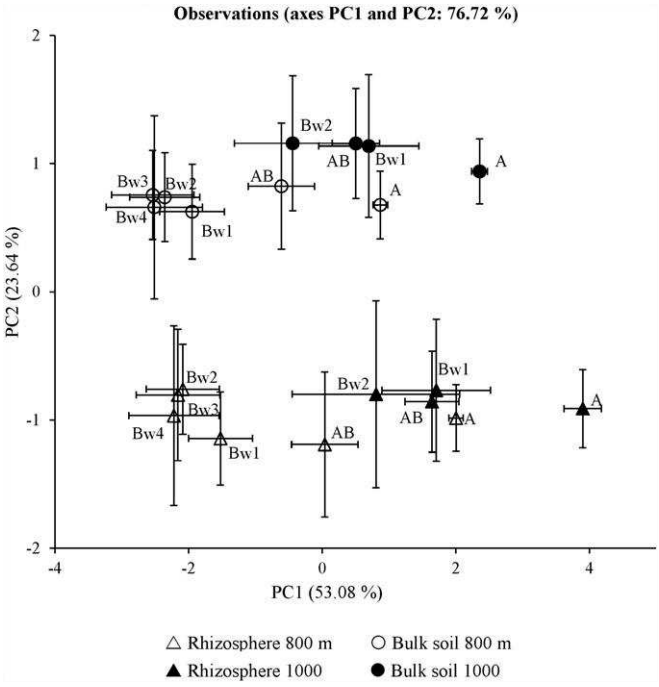

Fig. 1. Variation of rhizosphere and bulk properties for the soils under Fagus sylvatica forest at two altitudes (800 and 1000 m), as analyzed by principal component analysis (PCA) using standardized data. Central Apennines, Italy.

Table 2
Contributes of variables in PC1 and PC2 axes for the soils under study at Mt. Terminillo, Mt. San Vicino, and Mt. Acuto. Central Apennines, Italy.

Variables	PC1	PC2
	%	
pH in water	4.660	34.264
pH in KCl	8.802	23.118
TOC	15.841	2.363
WEOC	3.405	18.533
Total N	15.653	0.511
Organic P	12.026	5.162
Available P	12.718	4.610
Alkaline phosphatase	15.653	0.078
Acid phosphatase	11.331	11.360

3.2. Soil pH, organic C and total N

In the soils at both altitudes, pH_{H2O} and pH_{KCl} remained relatively constant throughout the profile, and no significant difference was observed between rhizosphere and bulk soil (Figure 2a, b). However, both pH_{H2O} and pH_{KCl} from A to Bw2 horizons were higher at 1000 m than at 800 m altitude.

As expected, TOC content tended to decrease with depth throughout all the soils (Figure 3a). On a horizon by horizon comparison between the two altitudes, TOC content was greater at 1000 m than at 800 m. Then, TOC concentration was always higher in the rhizosphere than in the bulk soil of the same horizon. In contrast to TOC, WEOC concentration was similar at 800 and 1000 m a.s.l. (Figure 3b); however, whereas for the soils at 800 m no difference in WEOC content occurred between rhizosphere and bulk soil, at 1000 m the rhizosphere had consistently a greater WEOC content than the bulk soil.

The total N content decreased with depth in all the soils and, as for the TOC, on a horizon by horizon comparison it was higher at 1000 than at 800 m (Figure 3c). Rhizosphere and bulk showed similar total N contents at both altitudes, with the exceptions of the Bw1 horizon of the soils at 800 m, where the rhizosphere displayed a greater total N content than the bulk soil.

3.3. Total, organic and available phosphorus contents, and phosphatase activities

The total and organic P content was much higher in the soils at 1000 m than in those at 800 m (Figure 4a, b). At both altitudes, rhizosphere and bulk soil generally did not show significant differences, with the exceptions of the Bw1 horizon for total and organic P, and Bw3 horizon for total P, always for the soils at 800 m. The available P content (Figure 4c) was also generally higher in the soils at 1000 m than in those at 800 m; further, in the higher altitude soils the rhizosphere had a greater concentration of available P than the bulk soil in all the horizons, while in the soils at 800 m this occurred in four over six horizons (A, AB, Bw2, and Bw3). The TOC:organic P ratio (Table S1 of the Supplementary Materials) showed a decreasing trend for both rhizosphere and bulk soil at 800 m, but it was not significantly different between the two altitudes and between the fractions, with the exception of the Bw4 horizon at 800 m where the rhizosphere showed a greater value than that of the bulk soil.

In the soils at 800 m, alkaline and acid phosphatase activities (Figure 5a, b) decreased with increasing depth from the A to the Bw1 horizons (Bw2 for acid phosphatase), to remain rather constant more in depth. At 1000 m, both enzymatic activities decreased from the surface to the AB horizon, to remain rather constant in the horizon underneath. By contrasting horizon by horizon, the alkaline phosphatase activity (Figure 5a) was greater in the soils at 1000 m, where small differences between rhizosphere and bulk were observed only in the Bw1 horizon. The acid phosphatase activity (Figure 5b) was similar in the A

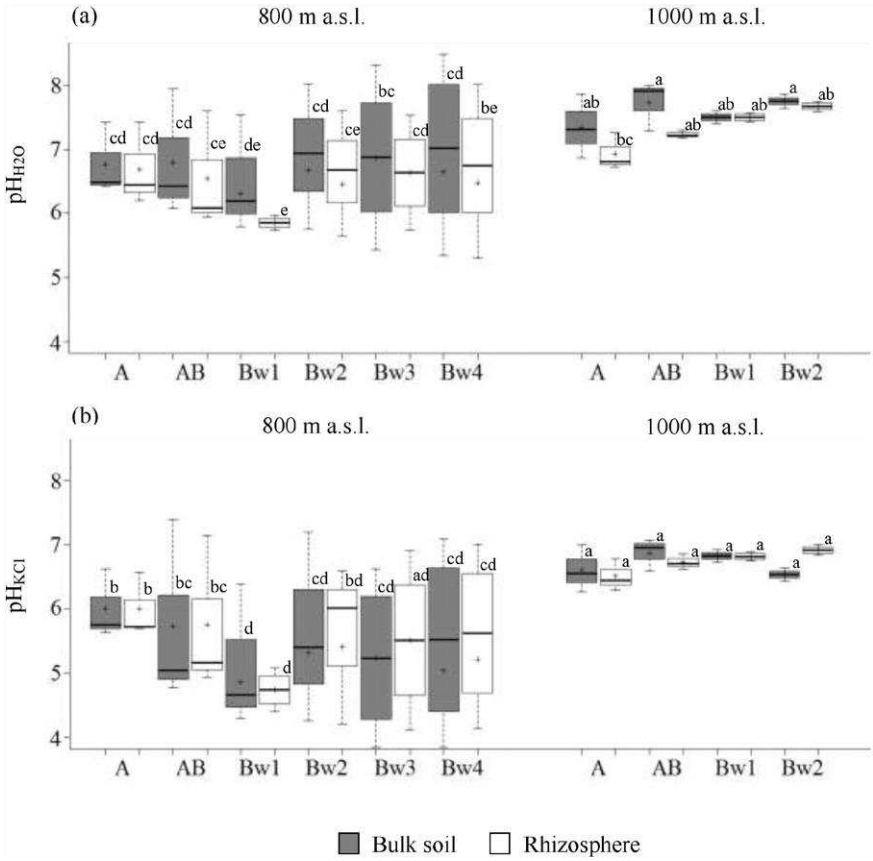

Fig. 2. Box plots showing the pH in water (a) and in KCl (b) of rhizosphere and bulk from soils under Fagus sylvatica forest at two altitudes (800 and 1000 m).Central Apennines, Italy. The + sign within each boxplot is the mean value, and different letters indicate significant difference at P b 0.05.

and AB horizons at the two altitudes, and greater at 1000 m than at 800 m in the Bw1 and Bw2 horizons. The rhizosphere showed higher acid phosphatase activities than the bulk soil in the Bw1, Bw2 and Bw4 horizons at 800 m, and in the A and Bw1 horizons at 1000 m.

3.4. ^{31}P NMR spectroscopy

The ^{31}P NMR spectra (Figure S3 of the Supplementary Materials) indicated that the orthophosphate monoesters (from 3 to 6 ppm of the chemical shift) were the dominating P forms of the spectral area, followed by inorganic orthophosphates (from 5.9 to 6.5 ppm), orthophosphate diesters (from −2 to 0 ppm) and pyrophosphates (from −3.5 to −5.5 ppm). The spectra showed similar patterns for rhizosphere and bulk soil. Between the two altitudes, the orthophosphate diesters were more abundant at 1000 m than at 800 m for both rhizosphere and bulk soil (Figure 6).

4. Discussion

4.1. Altitude and rhizosphere effect on pH, C and N

The PCA performed on all the measured soil properties showed a marked difference between rhizosphere and bulk of the studied soils, indicating that the beech roots induce a rhizosphere effect. Similar results were reported by several other authors. For example, Wang et al. (2001) studied the soil solution chemistry of beech and Norway spruce and found lower pH and nutrient concentrations in the rhizosphere than in the bulk soil solutions. Esperschütz et al. (2009), studying carbon fluxes from beech trees into the rhizosphere found that during the growing season the rhizosphere had a higher amount of dissolved organic matter than the bulk soil. Calvaruso et al. (2011) found that the rhizosphere of several tree species including beech was enriched in organic C, N, Ca, Mg and K with respect to the bulk soil, and the extent of the rhizosphere effect depended on the tree species. Finally, Cesarz et al. (2013) accomplished a rhizotron experiment and found that beech induced a strong rhizosphere effect mostly because of roots exudates and associated microbial community. Further, the PCA scoring plot (Figure 1) highlighted a clear distinction between the soils at 800 m and those at 1000 m, confirming the effect of altitude on soil properties, as commonly was reported by many authors (e.g., Tsui et al., 2004; Seibert et al., 2007; Cioci et al., 2008).

Even though the pH was the parameter that most strongly differentiated rhizosphere and bulk on the basis of PCA2, contrasting the two fractions for each horizon, no difference occurred for both pH$_{H2O}$ and pH$_{KCl}$. The weak rhizosphere effect on soil pH was ascribed, at least partly, to the sampling period as the reduced winter root activity probably lessened the release of H^+ at the soil-root interface in response to the lowered uptake of cations (Hinsinger et al., 2003). However, Calvaruso et al. (2014), in a study conducted on soil solutions of an acidic beech forest soil, measured lower pH values in the rhizosphere than in the bulk soil during winter, the reverse in spring, and similar pH values in the two fractions in fall. In our case, the scarcity of pH differences between rhizosphere and bulk could be also ascribed to the nature of the parent material, whose carbonate content makes the rhizosphere little susceptible to acidification as *i*) the carbonate dissolution neutralizes the activity of the released protons (Agnelli et al., 2016), and *ii*) the rate of chemical weathering and decarbonation in mountain soils is minor than at lower altitudes (Riebe et al., 2004). However, these observations support the higher pH values that we found for both rhizosphere and bulk of the soils at the higher elevation.

The TOC and total N content followed a similar decreasing trend with depth for both rhizosphere and bulk soil at the two altitudes.

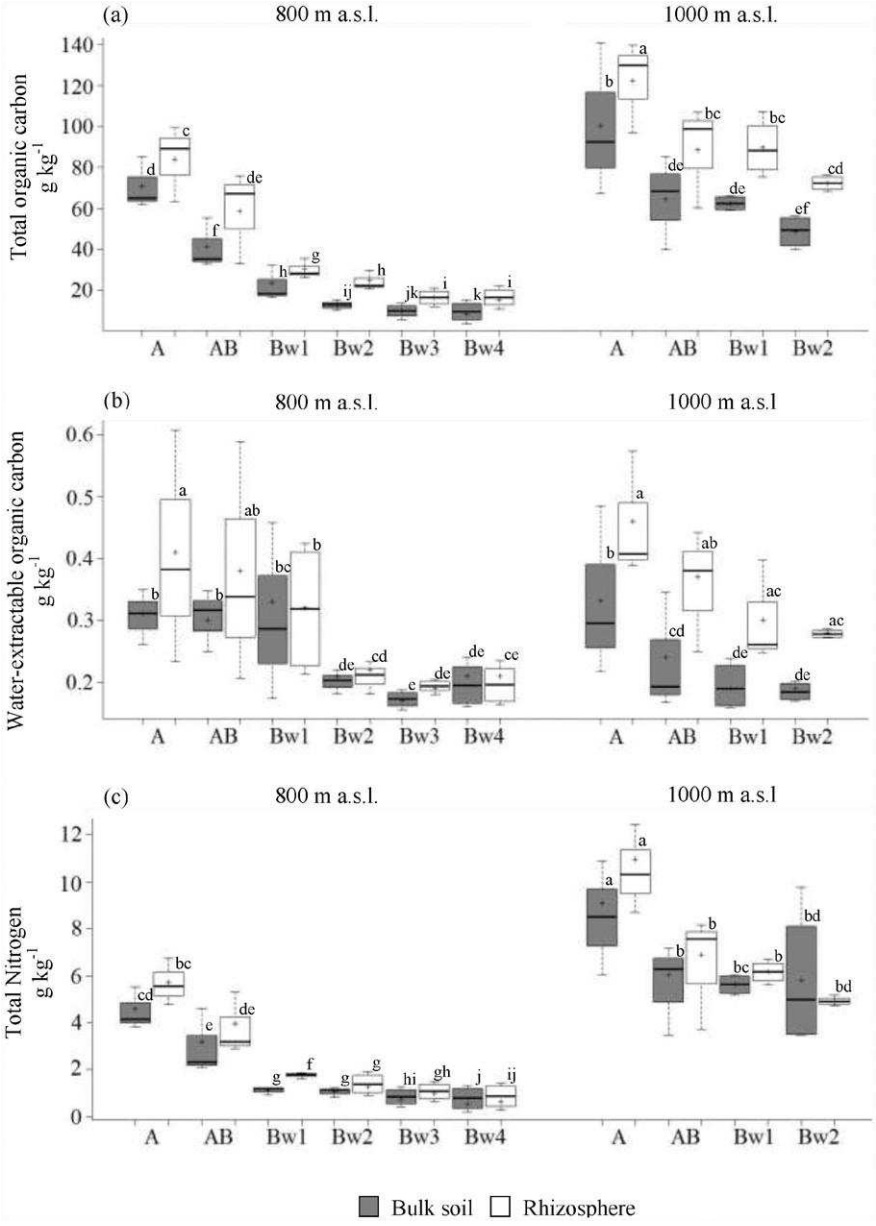

Fig. 3. Box plots showing the TOC (a), WEOC (b) and total N (c) content of rhizosphere and bulk from soils under Fagus sylvatica forest at two altitudes (800 and 1000 m). Central Apennines, Italy. The + sign within each boxplot is the mean value, and different letters indicate significant difference at P b 0.05.

This result was expected for soils with well-developed A horizons characterized by a strong crumb structure whose cement is mostly made of organics. According to several authors (e.g., Lemenih and Itanna, 2004; Dai and Huang, 2006; Follett et al., 2012), the higher content of TOC and total N at 1000 than at 800 m was related to climatic conditions and, among those, mostly to the temperature, which drops with increasing altitude; in contrast, the precipitation generally do not follow such a clear altitudinal trend (Körner, 2007; Griffiths et al., 2009). The accumulation of higher amounts of organic matter at 1000 m may have been fostered by the combination of the different effects caused by the lower temperature on the plant biomass production and on the activity of the soil microbial community. Although the plant biomass production decreases with increasing altitude (Zianis and Mencuccini, 2005), the higher amounts of TOC happens because of a lower microbial activity, which is due to colder soil temperatures occurring at higher elevations (Blume et al., 2002; Xu et al., 2014). In our study areas, a larger TOC content was found in the rhizosphere than in the bulk in the soils at both altitudes, as it has generally been observed in many different environments (e.g., Turpault et al., 2007; Zhao et al., 2010). As at 1000 m the rhizosphere had a larger WEOC content than the respective bulk soil in all the horizons, whereas this did not occur at 800 m, it suggested that beech was able to induce a stronger rhizosphere effect at the higher altitude. The enrichment of WEOC in the rhizosphere is mainly attributed to rhizodeposition processes (Chiang et al., 2006; Tuason and Arocena, 2009), which supply most of the energetic substrates for the rhizosphere microbial community (Koranda et al., 2011; Cesarz et al., 2013). According to Kuzyakov (2002, 2010), the availability of easily degradable compounds (and their consumption by the microflora) triggers a further mineralization of stable organic matter through the so-called “priming effect”. As a consequence, the rhizosphere priming effect induced by root exudation boosts the organic matter cycling and the microbially-mediated release of nutrients (Kaiser et al., 2011). This process may play a key role in the rhizosphere of the soils at higher altitude, where a scarcer soil development and a generally lowered microbial activity due to climatic constraints limited nutrients availabil-ity. The allocation of plant resources in the rhizosphere through

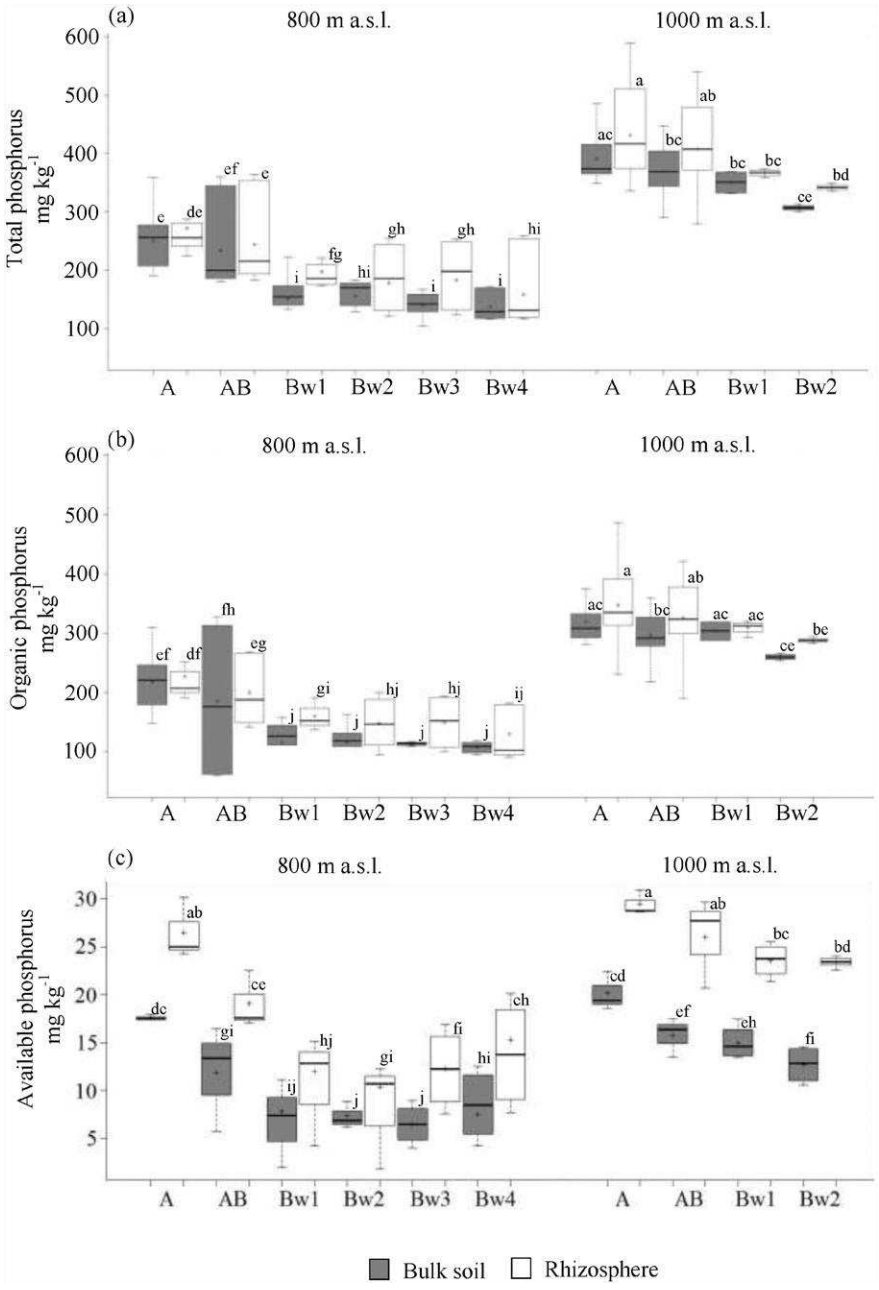

Fig. 4. Box plots showing the total (a), organic (b) and available (c) P content of rhizosphere and bulk from soils under Fagus sylvatica forest at two altitudes (800 and 1000 m). Central Apennines, Italy. The + sign within each boxplot is the mean value, and different letters indicate significant difference at P b 0.05.

rhizodeposition can therefore be seen as a strategy of the plants to overcome ecosystem (nutrient availability) restrictions (Boddy et al., 2008; Massaccesi et al., 2015). The fact that at 800 m the WEOC/TOC ratios for both rhizosphere and bulk were higher than those at 1000 m (data not shown), indicated a more active organic matter cycling occurring at the lower altitude, where the microbial activity has less limitations because of a milder temperature (Pietikäinen et al., 2005; Creamer et al., 2015).

4.2. Altitude and rhizosphere effect on P availability and related enzymatic activities

In agreement with many previous observations on forest soils, the total P content was mostly made up of organic P, which showed mean contents that are commonly reported for mountain soils (e.g., Makarov et al., 2004; Talkner et al., 2009). According to Turner et al. (2002) and Stutter et al. (2015), the greater concentration of organic P in the soils at 1000 m was attributed to the higher abundance of organic matter in these soils. The dependence of the organic P concentration on soil organic matter content was also confirmed by the TOC:organic P ratio, which showed no difference between the two altitudes and between rhizosphere and bulk soil, although this ratio could be also affected by the amount of available P (Makarov et al., 2004) and P plant uptake (Saikh et al., 1998).

As the organic P is the main source of available P in soil (Turner et al., 2014), the larger concentration of available P for both rhizosphere and bulk in the soils at 1000 m was ascribed to a greater alkaline phosphatase activity all throughout these soils, which was probably induced by the larger WEOC and TOC contents (Lemanowicz and Krzyzaniak, 2015; Stutter et al., 2015), and the higher pH of these soils, all factors able to promote the alkaline phosphatase activities (Nannipieri et al., 2011). For the Bw1 and Bw2 horizons, also the acid phosphatase activity

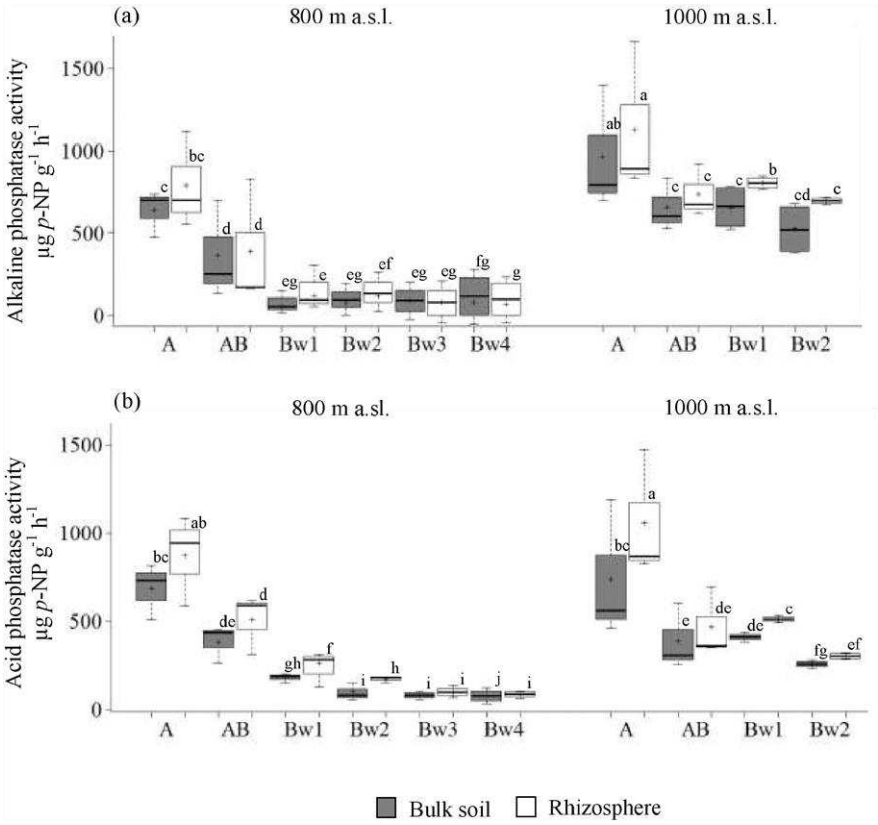

Fig. 5. Box plots showing the alkaline (a) and acid (b) mono-phosphatase activities of rhizosphere and bulk from soils under Fagus sylvatica forest at two altitudes (800 and 1000 m). Central Apennines, Italy. The results are expressed as µg of p-nitrophenol (p-NP) produced by 1 g of soil per h of incubation (µg p-NP g^{-1} h^{-1}). The + sign within each boxplot is the mean value, and different letters indicate significant difference at P b 0.05.

was higher in the soils at 1000 m, but in this case the fostering factors were probably only the high contents of WEOC and TOC. The question on whom, between plants roots and rhizosphere microbial community, was the main responsible for the different production of phosphatase in the soils at 800 and 1000 m remains open. Previous studies found that the acid and alkaline phosphatase activities are higher in the rhizosphere than in the bulk soil (Marschner et al., 2005; Zhao et al., 2007; Shi et al., 2011) because of rhizodepositions, which fuels the microbial activity and enhances the production of extracellular enzymes in the rhizosphere (Brzostek et al., 2013). However, also the plant roots produce enzymes (Nannipieri et al., 2011; Rejsek et al., 2012). To this regard, in a mesocosm experiment with young *Fagus sylvatica* L., Hofmann et al. (2016) found that plant phosphatases contributed lesser than microbial ones to the total phosphatase activity in P-rich soil. In our case, we hypothesized that the greater alkaline phosphatase activities found in the rhizosphere of the soils at 1000 m was due to the release from both beech roots and a specifically adapted microbial biomass. In the soils at 1000 m, the larger phosphatase activities and the higher

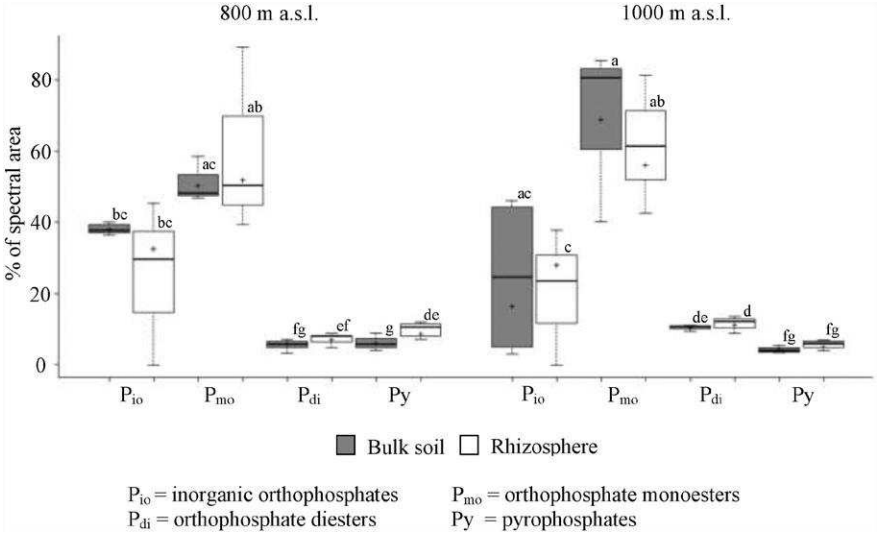

Fig. 6. Box plot showing the proportions of different P groups in NaOH-EDTA extracts from Fagus sylvatica forests at two altitudes (800 and 1000 m). Central Apennines, Italy. The + sign within each boxplot is the mean value.

availability of easily degradable organics (WEOC) probably counterbalanced the minor microbial activity caused by the lower temperature.

The mineralization of organic P compounds by hydrolysis of mononucleotides, sugar phosphates, phosphoproteins and inositol-phosphates via phosphatases is the process responsible for the release of inorganic orthophosphates, which are part of the available P and can be taken up by living organisms (Turner and Haygarth, 2005; Nannipieri et al., 2011). However, no substantial difference in phosphatase activities was found between rhizosphere and bulk soil at both altitudes. Because of this, the larger available P content in the rhizosphere of all the horizons of the soils at 1000 m was attributed to an intense P cycling that, in the soil close to the roots, was triggered by the exudation of labile organic compounds (Ström et al., 2002; Palomo et al., 2006); these would have promoted the microbial activity and the consequent release of P and other nutrients through the organic matter mineralization (Kuzyakov, 2010). Further, the higher content of available P in the rhizosphere compared to the bulk soil may be also favoured by the P uptake, which induces desorption of P from mineral surfaces (Gerke, 2015). However, a P solubilisation due to root release of protons following nutrient uptake and of organic acids cannot be excluded. This latter explanation would be valid even if no pH difference was detected between rhizosphere and bulk soil because of the buffering action of the calcareous parent material, and the complex spatial and temporal pat-tern of micro-niches occurring in the rhizosphere (Richter et al., 2007; Faget et al., 2013).

The hypothesis of a more intense P cycling occurring in the soil close to the roots was not supported by the results of the ^{31}P NMR analysis, as no significantly different proportions of P forms between rhizosphere and bulk soil were detected. This absence of differences between the fractions may be partly attributed to the soil variability (above and belowground) occurring even at the same altitude.

The most represented form of the P pools was that made of orthophosphate monoesters, either at 800 and 1000 m. This fact was rather expected as inositol-phosphates (which are the main component of the P monoesters) are strongly stabilized in soil by abiotic reactions with minerals, which thereby hinder their biological degradation and favour their accumulation in soil (Turner et al., 2002; Giaveno et al., 2010). The only difference between the sites showed by the NMR spectra was the larger proportion of orthophosphate diesters in the soils at 1000 m. As orthophosphate diesters are considered indicators of microbial P cycling (Stutter et al., 2015), their greater amount at 1000 m than at 800 m supports the occurrence of a general stronger P turnover in the soils at the higher altitude. This is consistent with the concept that high-altitude ecosystems, due to more pronounced nutrient limitations when compared to lower altitude ones, are more dependent on mineralization of soil organic matter by microbial community (Parfitt et al., 2005). Indeed, when the amount of available P in the soil is limited, P is largely immobilized in organic forms (Bünemann et al., 2012).

5. Conclusions

In this work we evaluated the rhizosphere effect of beech in forest soils of central Italy, at two altitudes (800 and 1000 m) and along 1° of latitudinal gradient. While the small latitudinal gradient did not affect the rhizosphere and bulk soil properties, significant changes occurred between the soils at the two altitudes, and a marked rhizosphere effect was detected in those at 1000 m. These differences were observed in spite of the spatial and morphological heterogeneity of the forest soils, which possibly affected the extent of the rhizosphere effect at the different altitudes. However, the fact that we tested our hypotheses in natural soils, where above and belowground heterogeneities are independent variables, may be considered as an added value to our research, and indicates that sampling designs able to control the main climatic and physiographic variables may allow obtaining significant results studying in vivo rhizosphere.

The clear rhizosphere effect that was found at the higher altitude and it was expressed by a greater TOC, WEOC and available P concentrations, was attributed to rhizodepositions, which represent the main source of energetic substrates for the rhizospheric microbial community. The greater availability of easily degradable compounds (WEOC) in the rhizosphere should boost the mineralization of organic matter, which in turn may favour the mineralization of the organic P forms and increase the amount of available P (Figure 7). Therefore, we speculated that at high altitude the energy supplied by the plants through

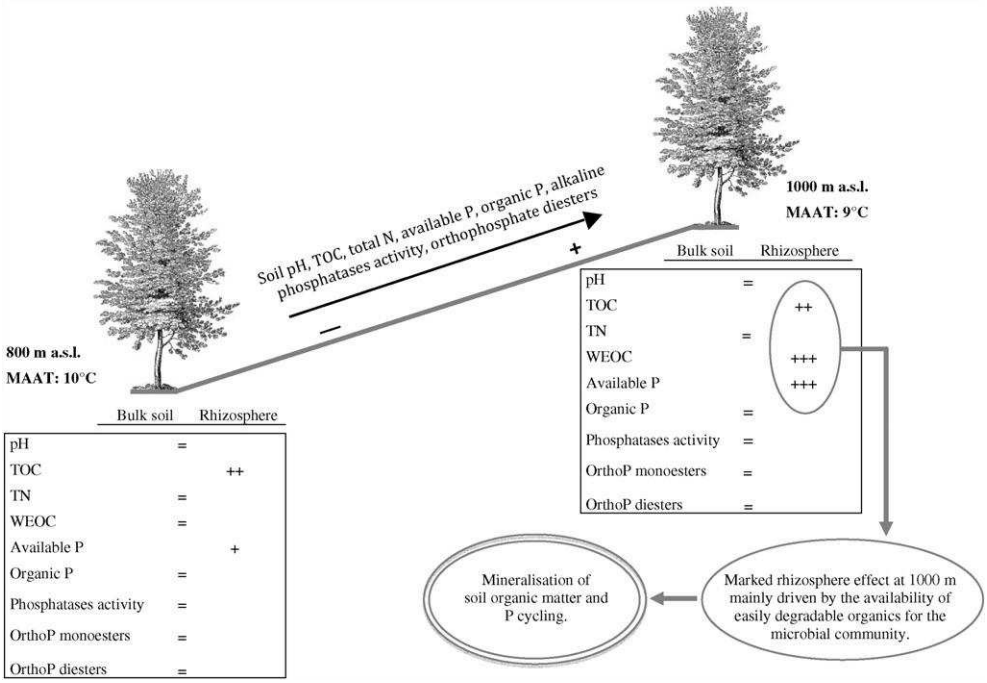

Fig. 7. Conceptual diagram of the altitude and rhizosphere effect on the soil properties of Fagus sylvatica forests at two altitudes (800 and 1000 m). Central Apennines, Italy.

rhizodeposition to the rhizosphere heterotrophic microbial community is key for fuelling the rhizospheric processes and, in particular, P cycling.

Our results suggested that an increase of the air temperature of about 1 °C, which is expected globally for the year 2050 (IPCC, 2013), and that is equivalent to the temperature shift between our study sites at 800 and 1000 m a.s.l., might cause a shortage of available P in the high altitude beech forest soils.

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Appendix A. Supplementary data

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Paper III

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"Altitude affects the quality of the water-extractable organic matter (WEOM) from rhizosphere and bulk soil in European beech forests"

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Abstract

Water-extractable organic matter (WEOM) is the most dynamic and bioavailable fraction of the soil organic matter pool. Although the litter floor is considered the main source of WEOM, roots release a great amounts of labile organic compounds through rhizodeposition processes which make the rhizosphere, the small soil volume in proximity to the root, a soil compartment enriched with WEOM. Since, both the rhizosphere and the labile organic C pool are highly sensitive to the environmental conditions, we evaluated the characteristics of WEOM from rhizosphere and bulk soil collected from the A horizons of European beech (*Fagus sylvatica* L.) forest soils of Apennines mountains (central Italy) at two altitudes (800 and 1000 m above sea level), using elevation as a proxy for temperature change. Specifically, we tested if 1) the water extractable C content is greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one 2) the amount of sugars and phenols of the WEOM are greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one; 3) the richness of compounds in the WEOM is greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one.

At both 800 m and 1000 m a.s.l., the main distinction occurring between WEOM from rhizosphere and bulk soil was due to the larger amounts of sugars in the soil close to the roots. Further, our results indicated an influence of the altitude on rhizospheric processes, as suggested by the larger concentrations of organic C and soluble phenols, and richness of tannin compounds in the rhizosphere WEOM than in the bulk soil at 1000 m. This influence has been attributed to climatic and soil constraints which enhanced the release of labile organics and secondary metabolites by rhizodeposition. As a whole, our data drew a picture where the roots were able to affect the characteristics of WEOM, and the environmental constraints, such as temperature, enhance the differentiation between rhizosphere and bulk soil. This view confirmed the influence of the rhizosphere on the soil C cycle, and the importance of the rhizospheric processes when the environmental conditions become limiting.

Keywords: rhizosphere effect; labile C pool; mountain soils; soluble phenols; climate change

1. Introduction

Water-extractable organic matter (WEOM) is a mixture of both macromolecules and low molecular weight compounds produced by decomposition of fresh (leaf litter, root exudates, decaying fine roots) and old soil organic matter (McDowell, 2003). This labile organic fraction, which accounts for a small portion of the soil organic matter (SOM), is the most dynamic and bioavailable fraction of the soil organic matter pool (McDowell, 2003). A key role on WEOM cycling is carried out by microorganisms which use these easily accessible molecules as their main source of energy

(Smolander and Kitunen 2002; Kaiser and Kalbitz, 2012) and, just because of the strong relationship with the soil microbial biomass carbon, WEOM is often also considered as an indicator of the microbial activity (Gutiérrez-Girón et al., 2015). Indeed, while the microbial respiration of organic substrates provides WEOM formation and loss of organic C (Bengtson and Bengtsson, 2007), the biodegradation of the labile organic molecules increases the proportion of stable organic matter forms due to the accumulation of less degradable compounds (Kalbitz et al., 2003).

Although the decomposition of the litter floor is considered the main source of WEOM (Kalbitz and Kaiser, 2007), roots release into the soil a wide range of labile and soluble organic compounds. In fact, plants can release into the soil environment 10 to 40 % of their total net C assimilation per year from their roots through respiration and rhizodepositions in forms of root exudates, mucilage, sloughed-off cells and decaying roots (Lynch and Whipps, 1990; Whipps, 1990; Bertin et al., 2003). The allocation of labile compounds through rhizodeposition processes makes the rhizosphere, the small soil volume in proximity to the root, a soil compartment enriched with WEOM compared to the bulk soil (Massaccesi et al., 2015; Agnelli et al., 2016). To this regard, Jia et al. (2015) found greater contents of carbohydrates and readily decomposable humic material in the rhizosphere than in bulk soil under citrus stands of 30 years old, while Fujii et al. (2012) detected a general larger concentration of organic acids in the rhizosphere than the bulk soil of tropical forests. Conversely to the rhizosphere, in the soil far from the roots the labile organics mainly derive from the decomposition of SOM by extracellular enzymes (Jones et al., 2009). The promptly decomposable organic compounds released by rhizodeposition processes fuel the rhizosphere heterotrophic microbial community (Boddy et al., 2007; Phillips et al., 2011), which in turn boosts the SOM decomposition by priming effect (Kuzyakov, 2002).

Since the several biogeochemical processes in which WEOM is involved and because of its high sensitivity to ecosystem disturbances, it is crucial to assess the impact of environmental changes (e.g., air and soil temperature) on the quantity and quality of WEOM. However, contrasting findings about the influence of the temperature on the WEOM properties are present in the literature: e.g., Xu et al. (2015) found that labile C and N pools increased with altitude in a subalpine *Abies faxoniana* forest soils located at high altitude in southwestern China; Gutiérrez-Girón et al. (2015) found a decreasing WEOM content with increasing altitude in Mediterranean mountain soils, while Hassouna et al. (2010) detected an irrelevant influence of temperature on the WEOM quantity in cropland soils. Further, Delarue et al. (2014) reported that the organic matter solubility increased at higher temperatures, Williams et al. (2016) found no variation on the amount of soluble phenols with temperature changes in pasture soils, and Roth et al. (2015) found that forest soils exposed to low temperatures had a greater richness of labile organic compounds than those exposed to warmer

conditions. The temperature has also an indirect effect on WEOM through an increase of organic C inputs into the soil via enhanced root exudation (Ultra Jr. et al., 2013; Yin et al., 2013a, b). The greater root exudation at higher temperature, in turn, decreases the pH values in the rhizosphere due to enhanced production of organic acids and CO_2 by microbial activity (Ultra Jr. et al., 2013). Thus, the roots of plants exposed to altered temperature may cause changes in the composition of the soil microbial community; to this regard, Zogg et al. (1997) incubated soil samples from a sugar maple forest at different temperatures and found that at high temperature the microbial community were able to metabolize substrates that were not usable by the microorganisms adapted at lower temperature.

The objective of the present study was to gain information on the influence of temperature on the quantity and quality of the WEOM pool in forest soils, and specifically in the rhizosphere, by using altitude as a proxy for temperature change (Vincent et al., 2014; Gutiérrez-Girón et al., 2015). With this aim, we contrasted the WEOM properties of rhizosphere and bulk soil of the A horizons of European beech (*Fagus sylvatica* L.) forests at two altitudes (800 and 1000 m) on three mountains in central Italy. It is noteworthy to say that the sites at 800 and 1000 m altitude had a mean annual temperature that differed for 1°C, which is the expected increase of the air temperature for the year 2050 (IPCC, 2013). Through the combination of chemical and spectroscopic analyses, we tested the following hypotheses: 1) the water extractable C content is greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one 2) the amount of sugars and phenols of WEOM are greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one; 3) the richness of compounds in WEOM is greater in the rhizosphere than in the bulk soil and at higher altitude than at lower one.

2. Materials and methods

2.1. Study areas

As study areas, three calcareous massifs were selected on the Apennines chain (central Italy): Mount Terminillo (42°28’ N, 12°59’ E), Mount San Vicino (43°19’ N, 13°03’ E), and Mount Acuto (43°28’ N, 12° 41’ E) (Figure S1 of the Supplementary Materials).

beech (*Fagus sylvatica* L.) forests were selected at about 800 and 1000 m above sea level, on slopes with an inclination ranging from 25° to 40° and northerly aspect. All the forests were coppices in conversion, with the conversion that started from about 20 to about 40 years ago. All soils developed from limestone and were classified according to Soil Survey Staff (2014) as Mollisols (Hapludoll or Haprendoll) or Inceptisols (Humudept). The mean annual air temperature (MAAT) in all the three study areas was 10°C at 800 and 9°C at 1000 m, with January as the coldest month and

July the warmest one. The mean annual precipitation was 1248 mm at Mount Terminillo, 825 mm at Mount San Vicino, and 1430 at Mount Acuto. A detailed description of each site was reported in De Feudis et al. (2016).

2.2. Soil sampling

During the late winter 2014, two profiles were opened within a plot of about 100 m^2 at each altitudinal site (2 profiles x 2 altitudes x 3 study areas). The winter sampling was chosen because the low rhizosphere and soil microbial community activities occurring during the cold season allowed to compare rhizosphere and bulk soil in a more stable condition rather than in dynamic seasons like spring or summer (De Feudis et al., 2016). The profiles were opened at about 50-60 cm from the stem of selected beech trees with an age ranging between 40 and 65 years old. The influence of the different age of the selected trees on the rhizosphere properties was negligible because we considered as rhizosphere only the soil associated to the fine roots, which activity is rather independent from the plant age because of their periodic turnover (Trumbore and Gaudinki, 2003; Agnelli et al., 2014).

The soils were morphologically described according to Schoeneberger et al. (2012), and a large amount of A horizon (at least 3 kg) was collected from each profile and stored in a portable refrigerator. The beech rhizosphere of each sample was isolated in the laboratory by picking up the roots with a diameter lesser than 2 mm together with the adhering soil (Cocco et al., 2013; Massaccesi et al., 2015). The roots with a greater diameter than 2 mm were discarded. After a gentle shaking to detach the weakly adhering soil particles, which were then added to the bulk soil (i.e. the soil not strictly adhering to the roots), the soil material firmly adhering to the fine roots was considered as rhizosphere and recovered by further shaking and soft brushing. Both rhizosphere and bulk soil samples were air-dried and sieved through a 2-mm mesh.

2.3. Soil chemical analysis

The soil pH was determined potentiometrically in water (pH_{H2O}) and in 1 M KCl solution (pH_{KCl}) (solid:liquid ratio of 1:2.5) after 30 minutes of stirring by a combined glass-calomel electrode.

The content of total organic C (TOC) was estimated by K-dichromate digestion, heating the suspension at 180 °C for 30 minutes (Nelson and Sommers, 1996), and total N content was determined by a Carlo Erba EA1110 dry combustion analyzer (Carlo Erba Instruments, Italy). Available P was estimated according to Olsen et al. (1954).

According to Alonso et al. (1998), phenols were extracted from the soil samples using a solvent mixture of methanol-distilled water (4:1 v:v ratio) with 2 % of triethylamine. Briefly, 5 mL of

solvent mixture were added to 1 g of soil sample, and shaken for 1 h with an orbital shaker (130 rpm). The suspentio was centrifuged at 1400 *g* for 10 min, and then filtered through a 0.45 µm membrane filter. The content of total phenols of the extracts was determined colorimetrically by the Prussian Blue method (Graham, 1992; Hagerman, 2002). Briefly, to a mixture composed of 0.1 mL of extract and 3 mL of distilled water, 1 mL of 0.016 M $K_3Fe(CN)_6$ (Prussian blue) solution was added, followed immediately by 1 mL of 0.02 M $FeCl_3$ + 0.1 M HCl solution; the mixture was put in a vortex for a while and then left to rest for 15 minutes. Then, 5 mL of stabilizer (600 mL of distilled water + 100 mL of 85 % H_3PO_4 solution + 100 mL of 1 % gum arabic solution) were added and the absorbance was read at 700 nm, by a Lambda EZ 150 UV/VIS Spectrometer (Perkin Elmer, USA), against a gallic acid standard. The total phenols concentration was normalized against TOC content and expressed as mg gallic acid equivalent g^{-1} organic C.

2.4. Cross Polarisation Magic-Angle Spinning Carbon-13 NMR Spectroscopy (^{13}C CPMAS NMR)

Solid-state ^{13}C NMR spectroscopy was employed to assess the main structural components of the SOM in the whole soil samples. Solid-state ^{13}C NMR spectra were acquired with the cross-polarization magic angle spinning (CPMAS) technique performed at 15 kHz, using a 7.05 T Varian INOVA^{TM} Unity (Varian Inc., Palo Alto, CA, USA) spectrometer at a frequency of 75.4 MHz. The spectra were collected with a sweep width of 25 kHz and an acquisition time of 20 ms. Contact time for cross-polarization and recycle delay were 1250 µs and 0.5 s, respectively. The ^{1}H radio frequency (RF) field strength was set to 47.0 kHz and the ^{13}C RF field strength to 41.1 kHz. An ascending ramp of 15.3 kHz on the ^{1}H-RF field was used (Berns and Conte, 2011).

2.5. Water-extractable organic matter

(WEOM) 2.5.1. WEOM extraction

The lack of a standardized method to obtain WEOM makes this organic material an operationally defined fraction as its composition depends on the combination of several factors: i.e., extracting solution (water or dilute saline solution), sample pre-treatment (fresh or air-dried) soil:solution ratio, time of contact, and energy of the treatment (Jones and Willett, 2006). For example, the organic matter obtained by a very mild extraction with a low ionic-strength aqueous solution, 1:2 solid: liquid ratio, short time of contact (1 min) and gentle stirring of the mixture is considered representative of soil solution dissolve organic matter collected in situ with zero-tension lysimeters (Zsolnay 2003; Chantigny et al., 2008). On the other hand, a more vigorous extraction using distilled water with wider solid: liquid ratio (1:5 or 1:10) and overnight shaking solubilizes greater

amount of organic matter thanks to partial aggregate disruption and release of organic moleculesthat are not typically in the soil solution (Kaiser et al., 2015). To this regard, since the goal of this study was not to study the effect of altitude on the dissolved organics in soil solution but to evaluate this effect on the whole labile and potentially soluble organic pool of rhizosphere and bulk soils, we chose to extract the WEOM by the following procedure (Agnelli et al., 2016): 1 g of rhizosphere or bulk soil sample was placed into a plastic container, submerged with distilled water (solid:liquid ratio 1:10) and shaken (140 rpm) overnight with an orbital shaker. The suspension was centrifuged at 1400 *g* f 10 min, and then filtered through a 0.45 μm membrane filter. The obtained solution was stored in the refrigerator and analyzed within a week.

2.5.2. *Chemical analyses*

The organic C content of the WEOM was analyzed by a TOC-5000A analyzer (Shimadzu Corp., Tokyo, Japan) on aliquots of the obtained extracted solution after the addition of a few drops of concentrated H_3PO_4 to eliminate inorganic C.

The contents of hexose and pentose sugars were colorimetrically estimated (Hofman and Dusek, 2003) by using the anthrone and orcinol methods, respectively. Briefly, for the hexoses, 5 mL of anthrone reagent (0.2 g of anthrone + 5 ml of ethanol + 95 ml of 75% H_2SO_4) were added to 1 mL of extracted solution in a shaking ice-water bath; once the mixture was cooled, it has been heated in a boiling water bath for 10 minutes and, then, cooled again in the ice-bath. Once cooled, the absorbance was measured at 625 nm against a glucose standard. For the pentoses, 3 mL of orcinol reagent [0.5 g of orcinol + 50 ml of ethanol + (0.18 g $FeCl_3 \cdot 6H_2O$ in 200 ml of concentrated HCl)] were added to 1 mL of extracted solution, and the mixture was heated in a boiling water bath for 20 minutes; once cooled, the absorbance was read at 672 nm using the ribose as standard. The soluble phenols concentration was determined colorimetrically by the Prussian Blue method.

2.5.3. Electrospray Ionization Fourier transform-ion cyclotron resonance mass spectrometry (ESI FT-ICR MS)

WEOM aliquots were characterised for their composition by ESI FT-ICR MS, which measures the mass-to-charge ratio of organic molecules after their ionisation. The electrospray ionization induces the breaking of the non-covalent bonds such as Van der Walls forces and electrostatic interaction, and typically allows the detection of molecules with a mass lesser than 1000 Da (Hertkorn et al., 2008; Stubbins and Dittmar, 2015). The ultra-high resolution in combination with exact mass characterisation (here below 1 ppm) of this technology allows to identify thousands of compounds for each sample (Sleighter and Hatcher, 2007). However, the FT-ICR MS cannot be used as quantitative analysis (Sipler and Seitzinger, 2008) because the polarity of the compounds affects the

intensity of the signals: higher the polarity, higher will be the intensity. To prevent quenching processes due to soil inherent permanent ions, a combined desalting and pre-concentration of the WEOM samples were performed by solid phase extraction (SPE, C18 hydra cartridges, Machery & Nagel, Düren, Germany), using methanol (≥99.98 % LC-MS grade; Carl Roth, Germany) as back eluent. The mean C recovery achieved with the SPE cartridges ranged between 66 and 71%, in agreement with the values reported in literature (e.g., Stubbins at al., 2012; Stubbins and Dittmar, 2015).

The ultra-high-resolution mass spectra were acquired using an ESI-LTQ-FT Ultra (ThermoFisher Scientific, San Jose, CA, USA) equipped with a 7 T supra-conducting magnet (Oxford Instruments, Abingdon, UK). The mass spectrometer was used in negative ionization mode with 2.9 kV spray voltage, -50 V capillary voltage, 275 °C transfer capillary temperature, and nitrogen as sheat gas.

The samples were introduced by a syringe pump providing an infusion rate of 8 µl/min, and full FT-ICR MS spectra between 200 and 1000 Da were recorded at a mass resolving power of 400.000 at m/z 400 Da. The spectra were averaged out of 7 scans, each scan is accumulated from 50 transients. Under adjusted cell filling of 5E5 in the presented investigations, combined with recalculations a mean deviation of 0.4 ppm was received. The formula assignment was performed by an in-house developed calculation program using Scilab routines.

2.6. Statistical analysis

Two-way ANOVA was carried out to assess the effect of altitude and soil fractions (rhizosphere and bulk soil) on the soil properties. The normality and homoscedasticity of the data were verified by graphical analysis of residuals and transformed if necessary. The transformation was selected by the maximum likelihood procedure suggested by Box and Cox (1964), as implemented in the boxcox function of the package MASS (Venables and Ripley 2002). The results presented and discussed are based on mean values and standard error, and the comparison of means was assessed by Tukey HSD post-hoc test ($P < 0.05$).

Principal component analysis (PCA) was applied only to the WEOM properties (contents of water-extractable organic C, pentoses, hexoses and phenols, and richness of lignins, tannins and condensed aromatic compounds) to identify the variables capable to explain most of the variability between the two altitudes and between the rhizosphere and bulk soil. The WEOM dataset was standardized prior the PCA by subtracting the mean and dividing by the standard deviation.

The data were analyzed using R software (R Core Team, 2014).

3. Results

3.1. Rhizosphere and bulk soil properties

The pH in H_2O and in KCl of the bulk soil showed a higher value at 1000 than at 800 m of altitude, while no difference occurred for the rhizosphere at 800 and 1000 m (Table 1). Within the same elevation, rhizosphere and bulk soil had similar pH values. At both altitudes, the TOC content was greater in the rhizosphere than in the bulk soil (Table 1). In contrast, the total N content was higher at 1000 than at 800 m, with no difference between rhizosphere and bulk soil (Table 1). The content of available P was similar at the two altitudes, but always with higher contents in the rhizosphere than in the bulk soil (Table 1). Total phenols concentrations, once normalized to the TOC content, showed no differences between rhizosphere and bulk soil, while a greater amount was found at 800 m than at 1000 m (Table 1).

Table 1. Main properties of rhizosphere and bulk from the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. Numbers in parentheses are the standard errors (n=3). For each line, mean values with different letters significantly differ for $P < 0.05$.

	800 m above sea level		1000 m above sea level	
	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil
pHH_2O	6.69 (0.29) b	6.77 (0.20) b	6.93 (0.14) ab	7.34 (0.15) a
pHKCl	5.98 (0.27) b	5.99 (0.18) b	6.51 (0.13) ab	6.60 (0.12) a
Total organic C (g kg^{-1})	81.11 (5.31) b	68.52 (4.22) c	119.66 (6.25) a	91.69 (8.98) b
Total N (g kg^{-1})	5.44 (0.58) b	4.36 (0.48) b	10.75 (0.85) a	8.76 (0.93) a
Available P (mg kg^{-1})	26.07 (1.92) a	17.29 (1.39) b	29.38 (0.75) a	20.09 (0.69) b
Total phenols-to-total organic C (mg gallic acid equivalent g^{-1} OC)	4.53 (0.27) a	4.59 (0.26) a	2.87 (0.13) b	3.10 (0.29) b

The CPMAS ^{13}C NMR spectroscopy detected six functional groups: alkyl-C like lipids, and cutin at 0–45 ppm, N-alkyl-C like amino acids at 45–60 ppm, O-alkyl-C such as carbohydrates, hemicellulose, and cellulose at 60–110 ppm, aryl-C such as lignins, tannins, and aromatic C compounds at 110–160 ppm, carboxyl-C like carboxyl acids at 160 – 190 ppm, and carbonyl-C such as aldehydes and ketons at 190–215 ppm. The relative concentration of these organic functional groups in the rhizosphere and bulk soils has shown the following general trend: O-alkyl-C > aryl-C ≥ ky -C > N-alkyl-C = carboxyl-C > carbonyl-C (Table 2 and Figure S2 of the Supplementary Materials). Rhizosphere and bulk soil showed no difference in the content of the various C forms, but some difference in the SOM structure occurred between the two altitudes: the

O-alkyl-C and the alkyl-C had a larger relative percentage at 800 m, the aryl-C showed greater proportion at 1000 m.

Table 2. Relative proportions of carbon containing functional groups as detected by CPMAS ^{13}C NMR analysis in rhizosphere and bulk from the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. Numbers in parentheses are the standard errors (n=3). Mean values with different letters significantly differ for $P < 0.05$.

Groups	800 m above sea level		1000 m above sea level	
	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil
	% of spectral area			
Alkyl-C	20.3 (0.5) d	20.7 (0.4) d	17.5 (0.4) e	17.0 (0.4) ef
N-Alkyl-C	13.5 (0.6) gh	13.9 (0.4) g	12.4 (0.3) gh	11.9 (0.4) gh
O-Alkyl-C	34.4 (1.2) a	32.7 (0.5) a	30.9 (0.2) b	30.4 (0.4) b
Aryl-C	19.8 (0.4) de	19.8 (0.4) de	24.6 (0.6) c	25.3 (0.7) c
Carboxyl-C	11.0 (1.0) h	11.9 (0.7) gh	12.6 (0.5) gh	13.1 (0.7) gh
Carbonyl-C	1.1 (0.2) k	1.1 (0.2) k	2.0 (0.2) jk	2.4 (0.5) j

3.2. WEOM extracted from rhizosphere and bulk soil

3.*2.1. Chemical and spectroscopic (ESI FT-ICR MS) analyses*

The water-extractable organic carbon (WEOC) concentration was similar at 800 and 1000 m above sea level (Table 3); however, whereas for the soils at 800 m no difference occurred between rhizosphere and bulk soil, at 1000 m the rhizosphere had higher WEOC content than the respective bulk soil.

The content of hexose and pentose sugars was always larger in the rhizosphere than in the bulk soil, although no significant difference between the two altitudes occurred (Table 3).

The concentration of soluble phenols was greater in the rhizosphere at 1000 m, while it did not differ between rhizosphere and bulk soil at 800 m (Table 3).

Table 3. Concentrations of water soluble organic C (WEOC), hexose and pentose sugars, and soluble phenols of soil rhizosphere and bulk from the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. Numbers in parentheses are the standard errors (n=3). For each line, mean values with different letters significantly differ for $P < 0.05$.

	800 m above sea level		1000 m above sea level	
	Rhizosphere	Bulk soil	Rhizosphere	Bulk soil
WEOC (g kg^{-1})	0.36 (0.04) ab	0.30 (0.02) b	0.44 (0.03) a	0.30 (0.04) b
Hexose sugars (g glucose-C kg^{-1}soil)	0.10 (0.02) a	0.04 (0.01) b	0.09 (0.01) a	0.05 (0.01) b
Pentose sugars (g ribose-C kg^{-1}soil)	0.05 (0.01) a	0.03 (0.01) b	0.06 (0.00) a	0.02 (0.00) b
Soluble phenols (g gallic acid equivalent kg^{-1} soil)	0.01 (0.00) b	0.01 (0.00) b	0.02 (0.00) a	0.01 (0.00) b

The analysis of WEOM by ESI FT-ICR MS produced a large data set with thousands of peaks corresponding to molecules with different mass-to-charge ratios (figure not shown). In particular, the data obtained by the mass analyses showed similar richness of compounds (formulas) in all rhizosphere and bulk soil samples of both altitudes (Table 4). Given the complexity of the data obtained from the ESI FT-ICR MS, the results were presented by plotting van Krevelen diagrams (Kim et al., 2003) of H/C versus O/C molar ratios of the CHO compounds (Hofmann et al., 2015).

Table 4. Number of CHO formulas as detected by ESI FT-ICR MS analysis, and relative proportion of lignins, tannins, condensed aromatics, and sugars of water-extractable organic matter (WEOM) of rhizosphere and bulk from the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. Numbers in parentheses are the standard errors (n=3). For each column, mean values with different letters significantly differ for $P < 0.05$.

	N° of formulas	Lignins	Tannins	Sugars	Condensed aromatics
		%			
800 m above sea level					
Rhizosphere	3403 (824) a	30.9 (3.5) a	15.0 (1.0) ab	3.6 (0.3) a	< 1%
Bulk soil	4114 (926) a	32.7 (3.1) a	9.8 (2.4) b	2.8 (1.1) ab	< 1%
1000 m above sea level					
Rhizosphere	2796 (94) a	33.4 (0.2) a	17.6 (0.9) a	3.4 (0.0) a	< 1%
Bulk soil	3928 (249) a	32.7 (3.3) a	13.0 (0.7) b	1.2 (0.2) b	< 1%

The diagrams (Figure 1) exhibited similar patterns for the different samples, with the molecules that dropped in the area characterized by H/C and O/C ratio of 0.2-2.0 and 0.1-1.0, respectively.

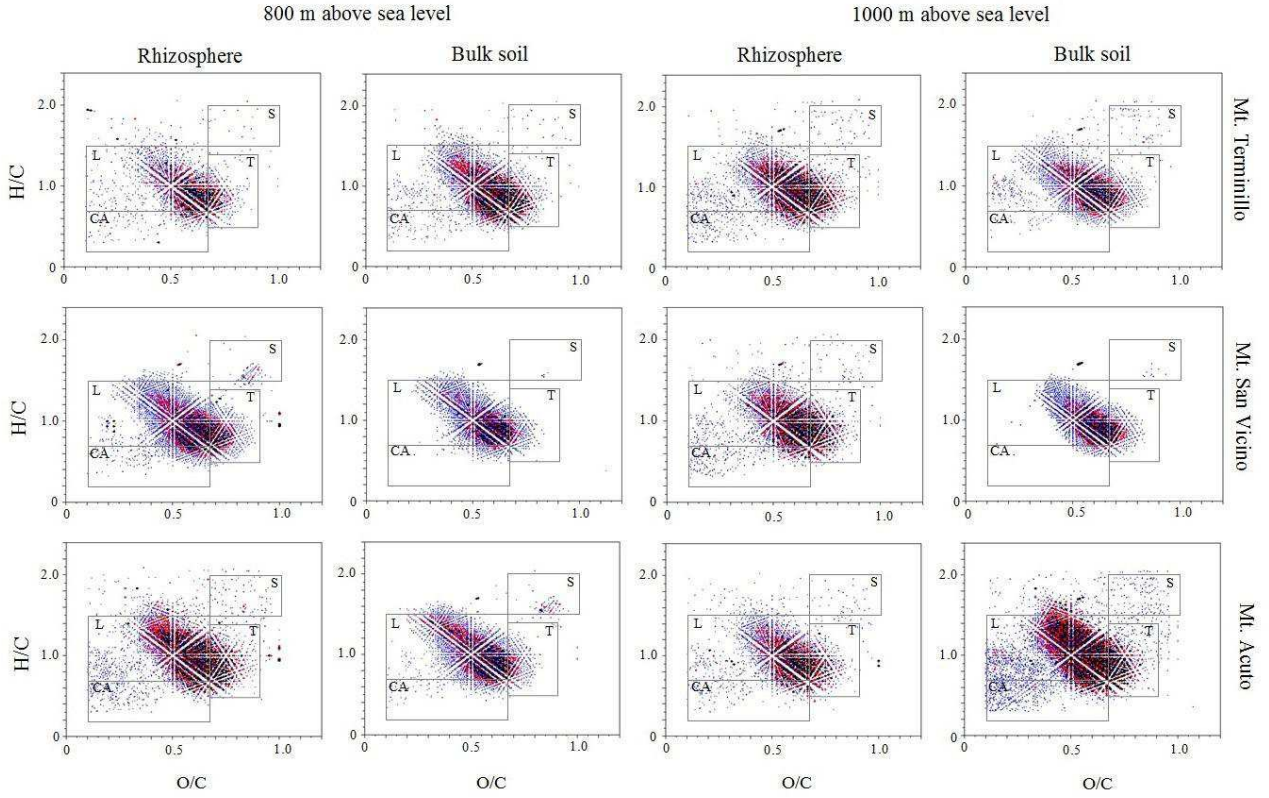

Figure 1. van Krevelen diagrams of H/C versus O/C molar ratios for the CHO compounds detected by the ESI FT-ICR MS in the water-extractable organic matter (WEOM) of rhizosphere and bulk from soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. Boxes overlain on the plots indicate the major biopolymer classes [lignins (L), tannins (T), condensed aromatics (CA), sugars (S)], whereas colors indicate relative mass peak intensity.

To estimate the proportion of the WEOM components of rhizosphere and bulk soil from the two altitudes, according to Sleighter and Hatcher (2007) and Ohno et al. (2010), four regions were delineated within the diagrams to cluster the detected molecules within the typical ranges of selected biopolymer: lignins (H/C: 0.7-1.5, O/C: 0.1-0.67); tannins (H/C: 0.5-1.4, O/C: 0.67-0.9); condensed aromatics (H/C: 0.2-0.7, O/C: 0.1-0.67); sugars (H/C: 1.5-2.0, O/C: 0.67-1.0). For all the samples, most of the molecules clustered in the regions corresponding to lignins and tannins (Table 4). However, while no difference was found between rhizosphere and bulk soil at 800 m, at 1000 m the rhizosphere showed a greater richness ($P < 0.05$) of compounds that fell in the tannins and condensed aromatics regions of the van Kravelen diagram (Table 4). The low proportion of sugars detected in the WEOM of all samples was attributed to the fact that the ESI FT-ICR MS weakly ionizes oxygen-rich molecules such as carbohydrates without permanent charge (Hertkorn et al., 2007). Furthermore, such high polar substances could be partly lost in the SPE process.

3.2.2. *Principal Component Analysis (PCA)*

The first two components calculated by PCA explained 64.38% of the total variance (Figure 2). The variability along the PC1 was mainly affected by the content of pentoses, hexoses and WEOC and the richness of tannins (Table 5). The variability along the second component (PC2) was mainly driven by the richness of lignins and condensed aromatic molecules (Table 5).

Table 5. Contributes of variables (%) and their correlation coefficients with PC1 and PC2 of the water-extractable organic matter (WEOM) from the soils under European beech (Fagus sylvatica L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy.

Chemical properties	PC1		PC2	
	%	Correlation coefficient	%	Correlation coefficient
Water-extractable organic C	17.96	0.71	3.65	-0.27
Pentoses	29.03	0.91	0.06	0.03
Hexoses	23.99	0.83	5.36	-0.33
Phenols	6.52	0.43	14.41	0.54
Lignins	6.40	-0.43	31.57	0.80
Tannins	13.84	0.63	15.14	0.55
Condensed aromatics	2.27	0.25	29.81	0.78

In the PCA scoring plot (Figure 2) the observations have been grouped (rhizosphere or bulk soil, 800 or 1000 m a.s.l.) and the 95% confidence ellipses were drawn for each group. The addition of confidence ellipses improved the interpretation of the scoring plot because they showed the degree of separation of the groups (Hammer and Harper, 2006; Montanari et al., 2016). The lack of overlapping of the confidence ellipses drawn for the WEOM from rhizosphere and from the bulk soil at 1000 indicated a distinction between the two groups. Conversely, this did not happen between the rhizosphere and bulk soil at 800 m. Further, the lack of overlapping of the confidence ellipses indicated a clear differentiation between the WEOM from the rhizospheres at the two altitudes.

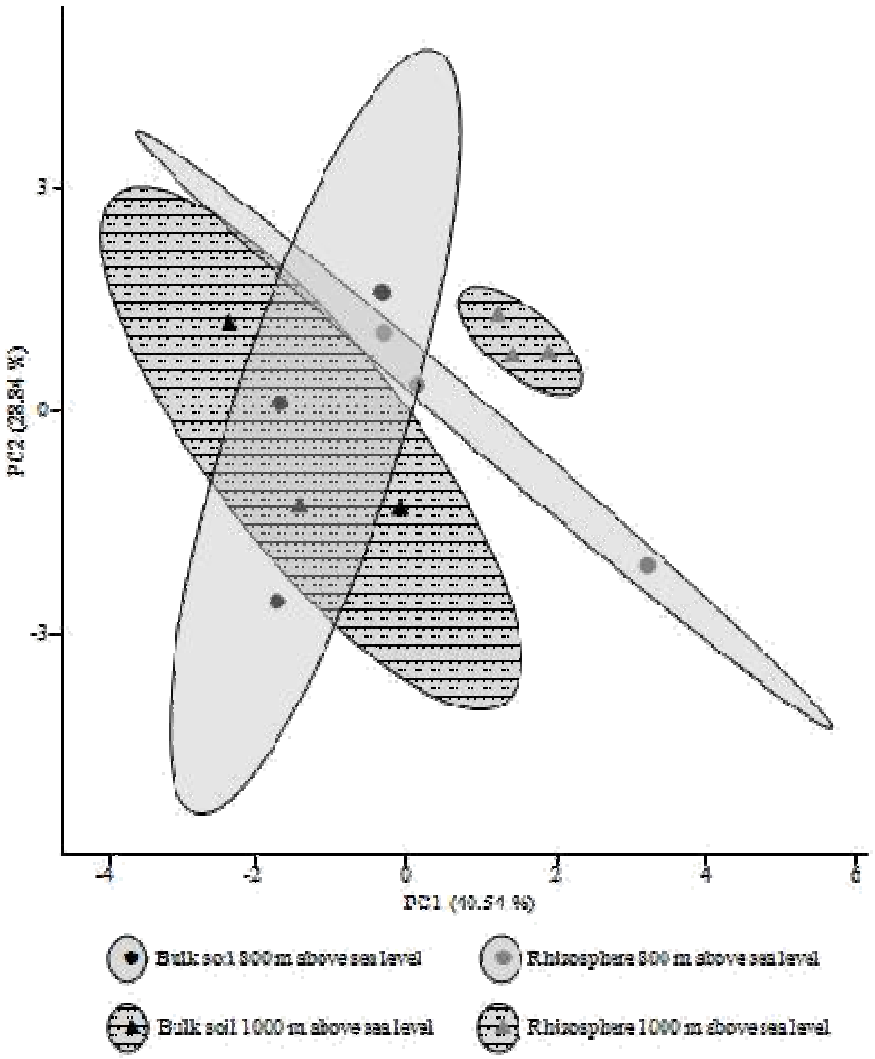

Figure 2. Variation of the properties of the water-extractable organic matter (WEOM) of rhizosphere and bulk from soils under European beech *(Fagus sylvatica* L.*)* forest at two altitudes (800 and 1000 m), as analysed by principal component analysis (PCA) using standardized data. Central Apennines, Italy. Also the 95% confidence ellipses are drawn.

4. Discussion

4.1. Chemical properties of rhizosphere and bulk soil at 800 and 1000 m of altitude

The pH values of the samples from both altitudes were near to neutrality, as reported by other studies on soils developed from alkaline substrates of central Italy (e.g., Corti et al., 2012; Cocco et al., 2013; Agnelli et al., 2016). However, the higher pH values of the bulk soil at 1000 than at 800 m were mainly ascribed to the lower weathering rate that occurred at higher altitudes (Riebe et al., 2004). Although the literature reported lower pH in the rhizosphere than in the bulk soil (e.g., Pandey and Palni, 2007; Cocco et al., 2013; Massaccesi et al., 2015), in our case the absence of significant differences between the two fractions were attributed to the calcareous nature of parent

material, that makes it little prone to acidification because the activity of the protons released by the roots are neutralized by the carbonate dissolution (Hinsinger et al., 2003; Richter et al., 2007; Agnelli et al., 2016). The greater TOC and total N contents at 1000 m than at 800 m confirmed the general positive correlation between organic matter content and altitude (Dieleman et al., 2013; Prietzel and Christophel, 2014; Tashi et al., 2016). Indeed, the lower temperatures occurring at higher altitudes, although reduce the net primary production and the litterfall inputs (Zianis and Mencuccini, 2005; Bu et al., 2012), cause the decline of the microbial activity (Yimer et al., 2006; Xu et al., 2014). The larger amount of TOC in the rhizosphere than in the bulk soil was mainly attributed to C input by root exudates, border cells and decaying roots (Sokolova, 2015).

The greater availability of P in the rhizosphere than the bulk soil at both altitudes was ascribed to the root activity (Hinsinger et al., 2009). Indeed, although soil P is limited because it is mostly part of stable organic compounds or in form of calcium- or iron-phosphates (Frossard et al., 1995; Nelson and Janke, 2007), plants are able to mobilize this element by the release of phosphatase enzymes (Nannipieri et al., 2011), and through exudation of low molecular weight organic acids (LMWOA), sugars and phenolic compounds (Carvalhais et al., 2011; Bowsher et al., 2015). In the same study sites, De Feudis et al. (2016), attributed the higher P availability in the rhizosphere to an enhanced organic matter cycling triggered by root exudation.

The chemical structure of the organic matter of rhizosphere and bulk samples, as revealed by the ^{13}C NMR spectroscopy, was similar to that found by previous studies on forest floors (e.g., Rumpel et al., 2002; Cepáková and Frouz, 2015), and this similarity was attributed to the strong influence of the above organic horizons on the underlying A horizon. In particular, the high proportion of O-alkyl-C among the C forms comprising the SOM was attributed to the incorporation of slightly decomposed organic residues (Rumpel et al., 2002) produced during the forest floor decomposition. The fact that O-alkyl-C compounds were the most represented in the ^{13}C NMR spectra of leaves and roots of beech (Angst et al., 2016), should support the general low degradation degree of the organic matter. However, the higher amount of O-alkyl-C at the lower altitude suggested a relative greater litter decomposition due to the higher mean temperature, which should have favoured a major incorporation of cellulose and hemicelluloses derived substances in the mineral horizons of the soils at 800 m than in those at 1000 m. In addition, the larger proportion of aliphatic compounds, resulting from microbial cells or from decomposition of humic acids (Mikutta et al., 2006), and the lesser amount of aromatic components, that are mostly part of lignin and humic substances (Golchin et al., 1997), confirmed the higher degradation degree of the organic pool at 800 m than at 1000 m. This fact should also support the higher concentration of total phenols found at lower altitude than at higher one. The results obtained from the ^{13}C NMR analysis, together with the TOC contents,

indicated that temperature affected both the quantity (through mineralization rate) and the quality of organic matter. Indeed, the low temperatures at the high altitude, besides to reduce the degradation rate of the organic matter, promotes the accumulation of aromatic substances, which further foster a greater organic C storage at 1000 than at 800 m. The NMR analysis did not reveal any difference between the SOM of rhizosphere and bulk soil, although some differences were expected. However, the lack of differences between the two fractions might be masked by the fact that we analysed the whole organic pool. Indeed, although one of the main rhizosphere effect is the enrichment of the soil close to the roots with labile C compounds (Angst et al., 2016), these represent a very little proportion of the whole soil organic carbon pool. In our case, the WEOC was less than 0.5% of TOC.

4.2. WEOM of rhizosphere and bulk soil at 800 and 1000 m of altitude

A higher content of WEOC in the rhizosphere than in the bulk soil occurred only at 1000 m, where harsher climatic condition and less soil development should induce large root exudation (Riebe et al., 2004; Augustin et al., 2015; Bowsher et al., 2015; Sokolova, 2015; Callesen et al., 2016). The greater release of labile substances and the following increase of the microbial activity, might have triggered the triggered the decomposition of recalcitrant organic compounds by "priming effect" (Kuzyakov, 2002),, with the consequent production of further labile molecules. However, similar concentrations of WEOC were found in the rhizospheres from the two altitudes. We explained the lack of significant differences between the rhizosphere at 800 and 1000 m as due to two counterbalancing processes: *i)* a general more effective decomposition of the whole organic matter pool fostered by the milder temperature at lower altitude (D'Amore et al., 2010; Toosi et al., 2014), and *ii)* a greater production of rhizodeposits at higher elevation (Dakora and Phillips, 2002; Chen et al., 2006; Guo et al., 2015). The different extent of the two processes, organic matter decomposition and rhizodeposition, occurring in the rhizosphere at the low and high altitude could be supported by the greater WEOC/TOC ratio in the rhizosphere at 800 m (0.47 % ± 0.03) than at 1000 m (0.38 % ± 0.03).

As supported also by the PCA (Figure 2), WEOM of the rhizosphere at 1000 m was qualitatively different from that of the respective bulk soil. In particular, the greater richness of tannins and the highest concentration of soluble phenols in WEOM of the rhizosphere than in that of the bulk soil at 1000 m (Tables 3, 4) were attributed to a response of the beech to restrictive environmental conditions (Kraus et al., 2003; Ni et al., 2013; Zhao et al., 2014) occurring at this altitude. The lower MAAT and the reduced pedological development of the sites at higher altitude (De Feudis et al., 2016) should have led plant roots to produce larger amount of phenolic compounds in their

exudates (Kraus et al., 2003; Kanerva, 2007). Conversely to the total soil phenols, which were more abundant in the rhizosphere than in the bulk soil but were not affected by elevation, the highest concentration of soluble phenols found in the rhizosphere at 1000 m can be considered as an index of the stronger rhizosphere effect occurring at higher altitude, as also suggested by the PCA (Figure 2). However, besides to a direct release by plant, the microbial degradation of organic substrates triggered in the rhizosphere by the higher amount of simple compounds should contribute to a further increase of the soluble phenols (Bardgett, 2005).

The richness of lignin-like and tannin-like compounds indicated a general presence of recalcitrant organic molecules (Almendros et al., 2000; Kögel-Knaber, 2002; Ohno et al., 2010) in the water extractable pool of the organic matter both in the rhizosphere and bulk soil. Indeed, these carboxyl-rich alicyclic molecules are considered as products derived from decomposition of organic materials and notable constituents of humic substances (Kraus et al., 2003; Hertkorn et al., 2006; Cesco et al., 2012; Buondonno et al., 2014). At higher altitude, the greater proportion of condensed aromatics in the rhizosphere than in the bulk soil (Table 4) should indicate the occurrence of intense humification processes that may have caused an increase of the content of these compounds (Bayer et al., 2002) in the vicinity of the roots. This hypothesis was supported by the higher richness in the rhizosphere at 1000 m of tannin-like compounds, which are recognized to play a key role in the humification processes (Kraus et al., 2003). Additionally, the higher number of condensed aromatic compounds in the WEOM of the rhizosphere at 1000 m could be partly due to the mobilization of humic substances promoted by the release through root exudation of LMWOA, which can break bridges between metals and humic moieties by complexing the metal cations (Takeda et al., 2009).

As for the soluble phenols, the higher content of sugars in the rhizosphere than in the bulk soil at both altitudes suggested that a source of these compounds was root exudation (Gunina and Kuzyakov, 2015). The release of sugars, as well as other low molecular weight organic compounds, is considered as a plant strategy to overcome environmental restrictions through the stimulation of the activity of the microbial biomass (Bowsher et al., 2015; Kaiser et al., 2015). However, we cannot exclude that the larger content of sugars in the rhizosphere was due to the activity of the microbial biomass which, besides to use these compounds as energetic substrate, is able to produce new carbohydrates both directly and following decomposition of plant residues (Gunina and Kuzyakov, 2015).

In addition to the above considerations, the different WEOM characteristics between rhizosphere and bulk soil at 1000 m might be also due to the activities of different microbial communities. For example, the meta-analysis conducted by Blankinship et al. (2011) revealed a higher microbial diversity in soils subjected to lower temperatures, while an increased fungal diversity in the

rhizosphere of an annual flowering plant at higher altitude was found by de Armas-Ricard et al. (2016).

4.3. Conclusions

In this work, we appraised the effect of altitude (800 and 1000 m) on some characteristics of WEOM extracted from the rhizosphere and bulk soil of beech forests in the Apennine mountains (central Italy). The main distinction occurring at both altitudes between the WEOM from rhizosphere and bulk soil was due to the larger amounts of sugars in the former than in the latter. The greater availability of these compounds in the rhizosphere WEOM was attributed both to rhizodeposition and microbial activity, and suggested that an intense organic matter cycling occurred in the soil close to the roots, with positive consequences on the nutrient mobilization. We hypothesized that the influence of altitude on rhizospheric processes, as revealed by the larger concentrations of organic C and soluble phenols, and richness of tannin compounds in the rhizosphere WEOM than in the bulk soil at 1000 m, was due to climatic and soil constraints which enhanced the release of labile organics and secondary metabolites by rhizodeposition processes (Figure 3). Further, the presence of these easily degradable compounds in the rhizosphere WEOM should boost the degradation of the stabilized organic matter as indicated by the higher richness of condensed aromatic molecules. Hence, environmental conditions can affect the belowground C allocation with potential impact on the forms and amounts of the rhizosphere labile organic C pool.

Because of its capability to reply to disturbances, the rhizosphere WEOM could be taken as an index of environmental changes following temperature variations in time rather than in space. However, more studies on WEOM and its composition are needed to strengthening this aspect.

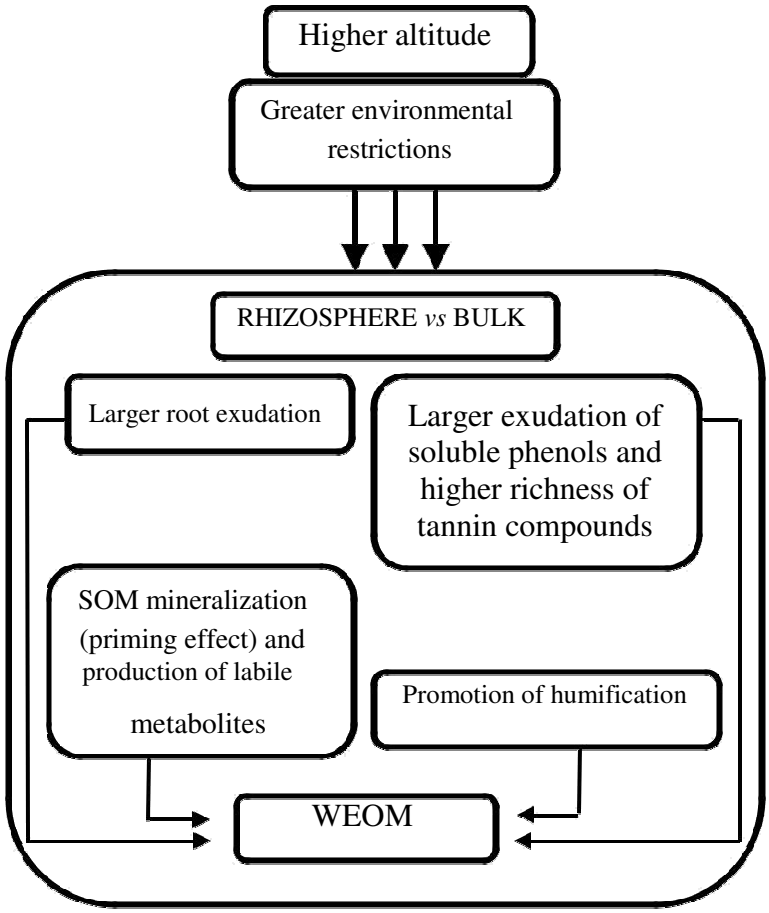

Figure 3. Schematic representation of the effect of the altitude on the quality of the water extractable organic matter (WEOM) of rhizosphere and bulk soils of European beech (*Fagus sylvatica* L.) forests at two altitudes (800 and 1000 m). Central Apennines, Italy.

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Supplementary materials

Figure S1. Map of Italy showing the location of the three study areas (Mt. Terminillo, Mt. San Vicino, and Mt. Acuto). Central Apennines, Italy.

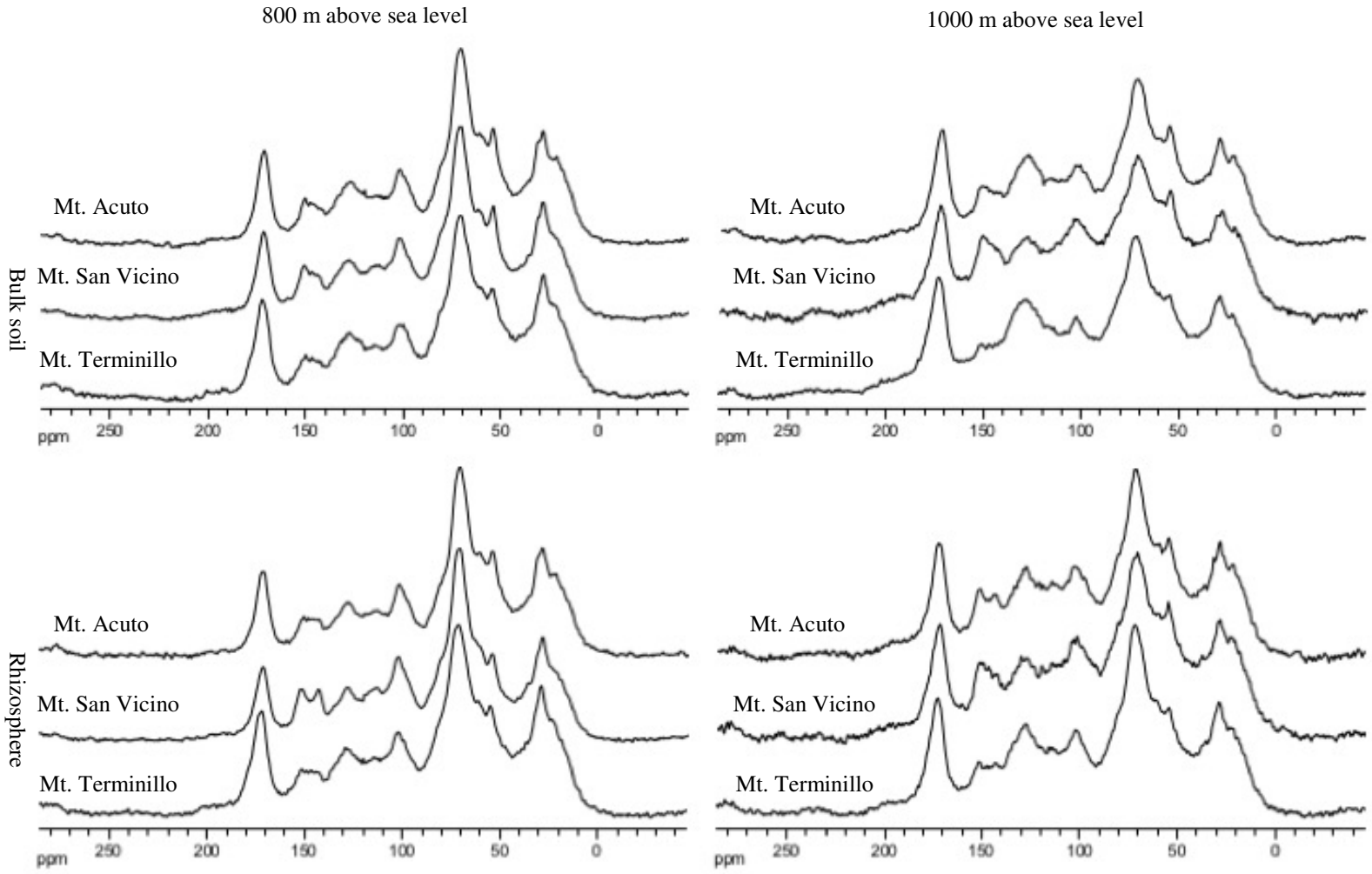

Figure S2. CPMAS ^{13}C NMR spectra of rhizosphere and bulk from the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy.

Table S1. General information and features for the soils under European beech (*Fagus sylvatica* L.) forest at two altitudes (800 and 1000 m). Central Apennines, Italy. For symbols see legend.

Horizon	Depth	Colour[a]	Structure[b]	Roots[c]	Skeleton
	cm				%

Mount Terminillo. Parent rock: grey limestone with small flintstone layers. Forest management: coppice in conversion to timber forest. Exposure: N. Mean annual precipitation: 1248 mm. Soil temperature regime: *mesic*. Soil moisture regime: *udic*.

Altitude: 800 m
Slope: 40°; Mean annual air temperature: 10.0°C; Mean temperature of the coldest month (January): 1.6°C; Mean temperature of the warmest months (July and August): 19.4°C. Vegetation: *Fagus sylvatica* L. (95%), *Acer* spp., *Laburnum anagyroides* Medik..

Soil: loamy-skeletal, mixed, mesic, Typic Hapludoll (Soil Survey Staff, 2014)

Forest floor	9-0	-	-	0	0
A	0-8	2.5YR 2.5/1	3f,m sbk, fr	3mi,vf,f,m,co	40
AB	8-16	2.5YR	2m pl→2f,m sbk, fr	3mi,vf,f,m,co	10
Bw1	16-23	5YR 3/4	2m pl→2f,m sbk, fr	2mi,vf,f; 3m,co	10
Bw2	23-33	5YR 4/4	2m pl→2f,m sbk, fr	2mi,vf,f,m,co	70
Bw3	33-66	5YR 5/4	1f,m abk, fr	2mi,vf,f,m,co	70
BC	66-77+	10YR 6/6	sg & 1f abk, fr	1mi,vf,f; 3m,co	70

Soil: loamy, mixed, mesic Lithic Haprendoll (Soil Survey Staff, 2014)

Forest floor	4-0	-	-	0	0
A	0-9	2.5YR 2.5/1	3f,m sbk, fr	3mi,vf,f,m,co	10
AB	9-19	5YR 2.5/2	3f,m sbk+abk, fr	2mi,vf,f,m; 3co	40
R	19-22+	-	-	-	-

Altitude: 1000 m
Slope: 40°; Mean annual air temperature: 9.1°C; Mean temperature of the coldest months (January and February): 1.0°C; Mean temperature of the warmest month (July): 18.3°C. Vegetation: *Fagus sylvatica* L. (99%), *Acer* spp..

Soil: loamy-skeletal, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014)

Forest floor	17-0	-	-	0	0
A	0-11	2.5YR 2.5/1	3f cr+sbk, fr	3mi,vf,f,m,co	20
AB	11-20	5YR 2.5/1	3f abk+sbk, fr	2mi,vf,f; 3m,co	40
BA	20-28	5YR 2.5/2	3f abk, fr	1mi,vf,f; 2m; 3co	50
2Bwb	28-38	5YR 4/3	1f,m abk, fr	1mi,vf,f,m,co	20
2Crb	38-49+	10YR 5/4	1f,m abk, fr	1mi,vf,f,m,co	75

Soil: loamy, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014)

Forest floor	16-0	-	-	0	0
A	0-14	2.5YR 2.5/1	3f,m sbk+abk, fr	3mi,vf,f,m; 2co	5
AB	14-24	5YR 2.5/2	3f,m sbk+abk, fr	2mi,vf,f,m,co	30
Cr	24-45+	10YR 6/4	1m abk, fr	2f; 3m,co	75

Mount San Vicino. Parent rock: grey limestone with marl and flintstone layers. Forest management: coppice in conversion to timber forest. Exposure: N. Mean annual precipitation: 825 mm. Soil temperature regime: *mesic*. Soil moisture regime: *udic*.

Altitude: 800 m
Slope: 35°; Mean annual air temperature: 10.0°C; Mean temperature of the coldest month (January): 1.0°C; Mean temperature of the warmest month (July): 21.0°C. Vegetation: *Fagus sylvatica* L. (90%), *Quercus cerris* L.*, Castanea sativa* Mill..

Soil: coarse-loamy, mixed, mesic, Typic Hapludoll (Soil Survey Staff, 2014)

Forest floor	9-0	-	-	0	0
A	0-10	7.5YR 5/2	2ccr	3mi,vf,f,m,co	10
AB	10-16	7.5YR 4/2	2f,m abk+sbk, fr	2mi,mf,f	20
Bw1	16-30	7.5YR 5/4	2f sbk, fr	2mi,vf,f	20
Bw2	30-43	10YR 4/3	2f, cr+sbk, fr	1mi,vf,f,m	20
2Bwb	43-68	10YR 4/2	2f cr, fr	3mi,vf,f; 1m	70
3Bwb	68-81	2.5Y 2/0	1f abk, fr	3mi,vf,f; 2m	70
3Crb	81-90+	7.5YR 4/4	1f abk, fr	1vf,f	90

Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2014)

Forest floor	7-0	-	-	0	0
A	0-9	7.5YR 5/2	2-3f cr, fr	3mi,vf,f,m	20
AB	9-16	10YR3/2	2f cr, fr	2mi,vf,f,m,co	30
Bw1	16-28	10YR5/4	2f sbk, fr	2mi,vf,f; 1m	40
Bw2	28-37	10YR3/4	3m sbk, fr	2mi,vf; 3f,m	20
2Crb	37-40+	10YR3/2	1f abk, fr	1mi,vf,f; 2m,co	80

Altitude: 1000 m
Slope: 45°; Mean annual air temperature: 9.0°C; Mean temperature of the coldest month (January): 0.0°C; Mean temperature of the warmest month (July): 20.0°C. Vegetation: *Fagus sylvatica* L. (80%), *Sorbus aria* Crantz (10%)*, Acer* spp., *Quercus cerris* L..

Soil: loamy-skeletal, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014)

Forest floor	6-0	-	-	0	0
A	0-7	5YR 2.5/2	3f cr, fr	3mi,vf,f,m,co	25
AB	7-16	5YR 3/3	3m,f cr+sbk, fr	3mi,vf,f,m,co	50
Bw	16-29	7.5YR 3/3	3f cr+sbk, fr	3mi,vf,f,m,co	70
2Bwb	29-40	2.5YR 3/4	3f sbk, fr	2mi,vf,f; 3m,co	25
2BCb	40-47+	10YR 6/5	1m abk, fr	1mi,vf,f; 2m,co	60

Soil: loamy-skeletal, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014)

Forest floor	4-0	-	-	0	0
A	0-5	5YR 2.5/2	3f sbk+cr, fr	3mi,vf,f,m	25
AB	5-14	5YR 3/2	3f cr+sbk, fr	3mi,vf,f; 2m	50
Bw1	14-36	5YR 3/3	2-3f,m sbk, fr	3mi,vf,f; 1m	70
Bw2	36-43	5YR 3/4	1m sbk, fr	2mi,vf,f; 1m	70
BC	43-52+	10YR 6/5	1m abk, fr	1mi,vf; 2f,m,co	70

Mount Acuto. Parent rock: white limestone with flintstone layers. Forest management: coppice in conversion to timber forest. Exposure: N-NE. Mean annual precipitation: 1430 mm. Soil temperature regime: *mesic*. Soil moisture regime: *udic*.

Altitude: 800 m
Slope: 40°; Mean annual air temperature: 10.0°C; Mean temperature of the coldest month (January): 0.0°C; Mean temperature of the warmest month (July): 21.0°C. Vegetation: *Fagus sylvatica* L. (95%), *Carpinus betulus* L.*, Acer opalus* Mill.*, Quercus* spp..

Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2010)

Forest floor	7-0	-	-	0	0
A	0-3	10YR 2/1	2-3f cr, vfr	2mi,vf,f	5
AB	3-9	10YR 4/4	3f,m sbk, fr	2vf,f,m,co	20
Bw1	9-25	10YR 5/4	1f,m abk+sbk, fr	3mi,vf,f 2m,co	30
Bw2	25-4	10YR 5/4	1m sbk+abk, fr	3mi,vf,f,m,co	30
Bw3	44-76	10YR 5/4	1m sbk, fr	2mi,vf,f; 3m,co	30
Bw4	76-99	2.5YR 5/6	1f,m sbk, fr	2mi,vf; 3f; 2m,co	50
Bw5	99-108	10YR 7/6	1m sbk, fr	1mi,fv,f,m,co	70
BC	108-115+	10YR 7/6	sg & 1f sbk, fr	1mi,fv,f,m,co	70

Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2014)

Forest floor	7-0	-	-	0	0
A	0-3	10YR 2/1	3f cr, fr	3mi,vf,f,m,co	10
AB	3-8	10YR 2/2	3f cr, fr	3mi,vf,f,m,co	10
Bw1	8-25	10YR 4/4	1f sbk, fr	3mi,vf,f,m,co	20
Bw2	25-38	10YR 4/4	1m,c sbk, fr	2mi,vf,f,m,co	30
Bw3	38-54	10YR 5/6	3f,m sbk, fr	2mi,vf,f,m,co	30
Bw4	54-73	10YR 5/4	1f,m sbk, fr	2mi,vf,f; 1m,co	40
Bw5	73-102	10YR 6/6	1f,m sbk+abk, fr	1mi,vf; 2f	30
BC	102-113+	10YR 7/6	sg & 1m sbk, fr	1mi,fv,f,m,co	50

Altitude: 1000 m

Slope: 25°; Mean annual air temperature: 9.0°C; Mean temperature of the coldest month (January): -1.0°C; Mean temperature of the warmest month (July): 20.0°C. Vegetation: *Fagus sylvatica* L. (100%).

Soil: coarse-loamy, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014)

Forest floor	5-0	-	-	0	0
A	0-11	7.5YR 3/2	3f,m sbk, fr	2mi,vf; 3f,m	10
AB	11-23	7.5YR 2.5/1	2f,m sbk, fr	2mi,vf,f; 3m,co	30
2Ab	23-33	7.5YR 3/2	3f,m sbk, fr	3mi,vf,f; 3m,co	50
2Bwb	33-54	10YR 7/6	1f abk, fr	2mi,vf,f	80
3Ab	54-63	10YR 4/3	1f sbk, fr	2mi,vf,f	60
3Bwb	63-70	10YR 5/3	1f abk, fr	2mi,vf; 1f	70
4Ab	70-71	7.5YR 3/2	3f abk, fr	1mi,vf,f	70
4Bwb	71-76	10YR 5/3	2f,m abk, fr	1mi,vf,f	75
4BC	76-86+	10YR 6/6	1f,m abk, fr	v1mi,vf,f	80

Soil: loamy-skeletal, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014)

Forest floor	13-0	-	-	0	0
A	0-13	7.5YR 2.5/1	3f,m cr+sbk, fr	3mi,vf,f,m; 2co	40
AB	13-20	10YR 3/2	1f,m sbk+abk, fr	2mi,vf,f,m,co	50
2Ab	20-36	10YR 2/2	1f abk, fr	2mi,vf,f; 3m,co	50
2Bwb1	36-69	10YR 4/4	1f abk, fr	2mi,vf,f; 1m	60
2Bwb2	69-75	10YR 6/6	1f abk, fr	1mi,vf	60
2BC2	75-85+	10YR 7/6	1f abk, fr	1mi,vf	75

[a]moist and crushed, according to the Munsell Soil Color Charts.

[b]sg=single grain; 1=weak, 2=moderate, 3=strong; f=fine, m=medium, c=coarse; cr=crumb, abk=angular blocky, sbk=subangular blocky; vfr=very friable, fr=friable.

[c]0=absent, v1=very few, 1=few, 2=plentiful, 3=abundant; mi=micro, vf=very fine, f=fine, m=medium, co=coarse.

Paper IV

Next submission

CHANGES OF TOPSOIL UNDER *FAGUS SYLVATICA* ALONG A SMALL LATITUDINAL-ALTITUDINAL GRADIENT

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Key words:
organic horizons, forest soils, soil organic matter, enzymatic activity, climate change.

Abstract

It is possible to hypothesize that warmer temperature influences litter decomposition and soil morphology and functionality in the same way it affects species distribution shifting polewards in latitude and upwards in elevation. We tested soil properties along a small transect in the Apennines chain (central Italy) at three different latitudes and, at each site, at two different altitudes (800 and 1000 m), with the intent to use latitudes and altitudes as surrogates of temperature gradient. Specifically, the study was conducted by contrasting chemical and biochemical (enzyme activities) parameters of the topsoil (O and A horizons) of European beech (*Fagus sylvatica* L.) forests at different scale of investigation: horizons, altitude and latitude. Along the topsoil profile, the trend of potential enzymes activity is peculiar of each horizon according to its degradation processes, availability of substrates, and nutrients. Indeed, the activity of all enzymes involved in the C-cycle (α-glucosidase, β-glucosidase, cellulase, xylosidase and glucuronidase), N-cycle (chitinase and leucine aminopeptidase) and some of the enzymes involved in the P mineralization (inositol-phosphatase, alkaline and acid phosphomonoesterase, phosphodiesterase) was the highest in the OLn horizons and decreased until the A horizon; this trend was ascribed to the input of fresh organic matter and the presence of macro and mesofauna. An inverse trend was recorded for arylsulphatase activity, which seemed to be active in a second phase of the organic degradation due to microorganisms involved in mineralization of S-compounds in the humic fraction. Pyrophosphatase activity also showed more affinity with the degree of organic degradation rather than with the content of organic C. Contrasting the altitudes of each sites, the difference of 1°C of temperature between 800 and 1000 m above sea level significantly affected some of the chemical and biochemical parameters such as the total organic carbon and total nitrogen, in greater amounts in the soils at 1000 m because of the colder temperature, and β-glucosidase and xilosidase activities, which were limited at higher altitude. A latitudinal sensitivity was recorded for β-glucosidase, xilosidase, cellulase, and acetate-esterase, although no difference in the mean annual air temperature occurred along the latitudinal transect. However, the summer-winter thermal excursion was the greatest for the northernmost site and decreased going south. Because of this, we hypothesize that wider thermal fluctuations may influence litter quality, C flux and, consequently, enzymes production by the microbial communities. Our approach allowed us to investigate the plant-soil relationship in the topsoil, and to make considerations on the effect of global warming in this type of ecosystems.

1. Introduction

Among several ecological consequences deriving from the temperature increase that are able to disturb the natural environments (Lindner et al., 2010), those affecting the relationship between soil and vegetation have been relatively little studied, although climate is recognized as one of the main factors controlling vegetation, litter degradation and soil formation (e.g., García-Palacios et al., 2013). According to Aerts (2006), climate has hierarchical control levels on litter degradation both directly and indirectly. Through changes in soil temperature and moisture, climate directly alters the rates of litter mass loss due to the high sensitivity of biological processes to temperature and water availability, and affects the composition of vegetation, plant litter quality, and structure of decomposer and detritivore communities. Consequently, it could be hypothesized that warming temperature will influence litter decomposition and soil morphology and functionality in the same way it affects species distribution shifting polewards in latitude and upwards in elevation (Corlett and Westcott, 2013; ICCP, 2007; Lenoir et al., 2008; Lindner et al., 2010; Peñuelas et al., 2007), with a re-arrangement of the ecological equilibria occurring between organic and mineral soil horizons. One of the main role in the decomposition processes is played by microbiota and its enzymatic pool (Aerts, 2006; Kourtev et al., 2002). With the physical and chemical breakdown of plant and animal residues, soil macro-, meso- and micro-organisms are able to transform litter into inorganic molecules and humic substances (Sinsabaugh et al., 2002; Swift et al., 1979). Thus, soil microbial activity is intimately linked to litter degradation, a fundamental process to maintain the ecosystem functions. Indeed, plant biomass production is supported by organic matter mineralization and humification processes, which allow elements cycling ensuring nutrients to plants (Almagro et al., 2015; Kavvadias et al., 2001; Mincheva et al., 2014; Wang et al., 2015). The activity of the soil microbial communities is influenced by soil type, vegetation, litter quality, and environmental factors. All these factors affect type and size of the soil microbial communities, which produce enzymes that modify the substrate and may lead to a community succession (Sinsabaugh et al., 2002). Thus, enzymatic activity can be used as an indicator of soil changes (Allen et al., 2011; Schloter et al., 2003) because it is controlled, beyond biotic processes, by abiotic factors such as atmospheric warming and precipitation patterns (Burns et al., 2013). As reported by Lu et al. (2014), changes in temperature can increase, decrease or not affect soil microbial activity. For this reason, the evaluation of the enzymatic activity may reveal the microbial substrate use efficiency and the extent of the nutrient cycling in different environments. In their review, Burns et al. (2013) reported that the change of soil temperature, atmospheric carbon dioxide concentration, and

frequent wetting-drying cycles affect enzyme activity, directly or through stimulation of plant growth and litter deposition. To investigate the effect of temperature on soil morphology and chemical and biochemical processes, we followed a sampling strategy able to mimic a temperature gradient by using latitude and altitude (Vincent et al., 2014). Following this rational, we selected three mountain sites on the Apennines chain (central Italy) at three different latitudes. At each site, we chose two areas at different altitude (800 and 1000 m) with similarly managed forests of European beech (*Fagus sylvatica* L.), the most diffused tree in the Mediterranean mountains. The small transect, in altitude and latitude, was consciously selected to evaluate the effect of 1°C of temperature change between altitudes and to minimize the environmental differences among sites. The study was conducted on the topsoil, the soil portion represented by the O (organic, formed by decaying litter) and A (mineral) horizons.

Specifically, the aim of this work was to establish the effect of latitude and altitude on the soil chemical and biochemical characteristics at three different detail levels: *i*) microscale, by contrasting soil horizons; *ii*) mesoscale, by contrasting soils at different altitude; *iii*) macroscale, by contrasting soils at different latitude. As altitude and latitude were taken as surrogates of potential temperature changes, we assessed and speculated on the effect of these changes on litter decomposition, soil morphology and soil functionality.

2. Materials and methods

2.1. Study sites

Three study sites with similar lithology, physiography, forest management, and vegetation were selected along a South-to-North transect of 1° of latitudinal gradient on central Apennines (Italy): Mount Terminillo (MT) (42°28’ N, 12°56’ E), Mount San Vicino (MV) (43°19’ N, 13°03’ E) and Mount Acuto (MA) (43°28’ N, 12° 41’ E) (Figure 1). At each site, two areas were selected on the North-facing slope, at about 800 and 1000 m above sea level (a.s.l.). According to the climatic data recovered from Centro Appenninico "C. Jucci" (2015) for MT (1956-2014 series), and from Spina et al. (2006) and Amici e Spina (2002) for both MV and MA (1950-2000 series), the two areas at 800 and 1000 m a.s.l. had a mean annual air temperature (MAAT) of 10 °C and 9 °C, respectively. At the three sites, the soils developed from limestone and were classified as Mollisols or Inceptisols (Soil Survey Staff, 2014). The wood was a mesophylic beech (*Fagus sylvatica* L.) forest in conversion to timber forest. The plant composition formed a *Polysticho-Fagetum* vegetal association (IPLA, 2001), with a

strong dominance of beech. A summary of the environmental conditions of each site is reported in Table 1.

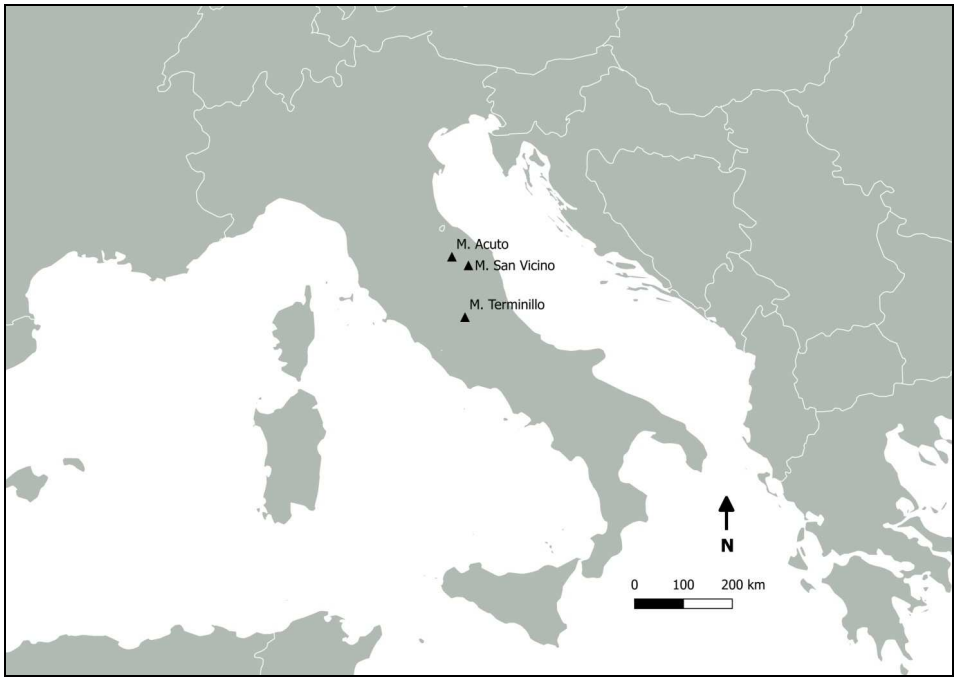

Fig. 1 - Map indicating the study sites

2.2. Soil sampling

The samples were collected during March-April 2015. To assess the spatial variability of the litter and its properties, 5-6 pits were dug at each site. On the basis of these observations, two topsoil profiles representative of the area conditions were opened at both altitudes. Minimum distance between the two topsoil profiles was 8 m, the maximum was 14 m. Each topsoil profile was opened at 50-60 cm from the stem of a beech and morphologically described following both Schoeneberger et al. (2012) and Baize and Girard (2008) for the O horizons, and per Schoeneberger et al. (2012) for the A horizons. Morphological observations of the topsoil profiles are given in Table 1.

Both O and A horizons were collected by using a 40 x 40 cm sampling frame. For each O horizon, all the material inside the frame was collected; the same was done for the A horizon. Depending on the horizon thickness, the sampled amount for the O horizons ranged from 270 to 910 g (air-dry weight); for the A horizons, the whole sample was homogenized in the field and an aliquot of at least 2 kg was collected. In the field, all the samples were stored in a portable refrigerator. Once in the laboratory, a quarter of each sample was stored at 4°C for enzymatic analyses, while the rest was allowed to air-dry. Thus, about one half of each dried O sample was left in natural conditions, while the other half was ground until it passed

Table 1. General features and morphology of the topsoil under *Fagus sylvatica* L. at 800 and 1000 m of altitude for mountains of central Apennines (Italy) within 1° of latitudes. For symbols see legend.

Horizon[a]	Horizon[b]	Depth	Colour[c]	Structure[d]	Roots[e]	Mycelia[f]	Small Macrofauna[g]	Mesofauna[h]	Boundary[i]	Thickness	Other observations
		cm								cm	
Mount Terminillo. Parent rock: grey limestone with small flintstone layers; Forest management: coppice in conversion to high forest.											
Altitude: 800 m											
Slope: 40°; Exposure: N; Mean annual precipitation: 1248 mm; Mean annual air temperature: 10.0°C; Winter mean temperature: 2.1°C; Spring mean: 7.9°C; Summer mean temperature: 18.9°C; Autumn mean temperature: 11.3°C. Drainage class: Moderately well drained. Vegetation: *Fagus sylvatica* (95%), *Acer* spp., *Laburnum anagyroide;* 25% of soil cover made by: *Hedera helix, Luzola sylvatica, mosses, Viola* spp., *Clematis vitalba, Juniperus communis Daphne laureola, Lamium purpureum, Fagus* seedlings. Soil: loamy-skeletal, mixed, mesic, Typic Hapludoll (Soil Survey Staff, 2014).											
Oi	OLn	10-7	-	-	0	0	+	(+)	cw	2-4	Beech cupules, leaves, twigs and bark. Few *Quercus* leaves.
Oi	OLv1	7-4	-	-	0	0	+	+	cw	2-3	Beech cupules, leaves, twigs and partially degraded bark, beechnuts.
Oi	OLv2	4-2	-	-	0	+/++	++	++	cw	2-3	Beech cupules, leaves, twigs, and partially degraded bark, beechnuts.
Oa	OHr	2-1	5YR 2.5/2	-	3mi,vf,f,m	+++	+++	+++	cw	1	Recognisable only beech cupules.
Oa	OHf	1-0	5YR 2.5/1	-	3mi,f,m	+++	++	++	cw	1	Darkish beechnuts.
A	-	0-9	2.5YR 2.5/1	3f,m sbk, fr	3mi,vf,f,m,co	0	(+)	+	cw	7-10	Skeleton: 40% (centimetric dimension, 5-6 cm).
Soil: loamy, mixed, mesic Lithic Haprendoll (Soil Survey Staff, 2014).											
Oi	OLn	6-3	-	-	0	0	+	(+)	cw	2-3	Beech stipels, leaves, twigs and bark, beechnuts. Few *Quercus* and *Acer* leaves.
Oi	OLv	3-1	-	-	0	+++	+	+	cb	2-3	Beech stipels, leaves, twigs, and partially degraded bark, beechnuts. Few *Acer* samaras. Skeleton (5%).
Oa	OHr	1-0	5YR 2.5/2	-	3mi,vf,f,m	+++	+++	++	cb	1-2	Skeleton: 10%. Darkish beechnuts.
A1	-	0-8	2.5YR 2.5/1	3f,m sbk, fr	3mi,vf,f,m,co	0	(+)	+	cw	5-9	Skeleton: 10% (centimetric dimension, 4-5 cm).
A2	-	8-17	5YR 2.5/2	3f,m sbk+abk fr	-	0	(+)	(+)	cw	8-11	Skeleton: 40% (centimetric dimension, 6-10 cm).
Altitude: 1000 m											
Slope: 40°; Exposure: N; Mean annual precipitation: 1248 mm; Mean annual air temperature: 9.1°C; Winter mean temperature: 1.3°C; Spring mean temperature: 7.2; Summer mean temperature: 17.7°C; Autumn mean temperature: 10.1°C. Drainage class: Moderately well drained. Vegetation: *Fagus sylvatica* (99%), *Acer* spp.; 5% of soil cover made by: *Fagus* seedlings. Soil: loamy-skeletal, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014).											
Oi	OLn	17-15	-	-	0	0	+	(+)	cw	1-3	Beech cupules, leaves, twigs and bark.
Oi	OLv1	15-10	-	-	0	++	+	++	cw	2-6	Beech cupules, leaves, twigs and bark, beechnuts.
Oi	OLv2	10-6	-	-	0	+++	++	++	cw	2-5	Beech cupules, leaves, twigs and partially
Oi	OLv3	6-4		-	1mi,vf	+++	+++	++	cw	1-3	70% of recognisable material. Brownish beechnuts.
Oa	OHr	4-0	10R 2.5/2	-	3mi,f,m	++	+++	++	cw	3-5	Skeleton: 5% (centimetric dimension, 3-4 cm). Darkish beechnuts.
A1	-	0-11	2.5YR 2.5/1	3f cr+sbk, fr	3mi,vf,f,m,co	0	(+)	+	cw	10-13	Skeleton: 20% (centimetric dimension, 4-6 cm).
A2	-	11-20	5YR 2.5/1	3f abk+sbk, fr	2mi,vf,f; 3co	0	(+)	(+)	cw	7-10	Skeleton: 40% (centimetric dimension, 5-8 cm).
A3	-	20-29	5YR 2.5/2	3f abk, fr	1mi,vf,f; 2m; 3co	0	(+)	(+)	cw	8-14	Skeleton: 50% (centimetric dimension, 7-8 cm)

Soil: loamy, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014).

Oi	OLn	16-11	-	-	0	0	(+)	(+)	cw	3-6	Beech cupules, leaves, twigs and bark. Few *Acer*
Oi	OLv1	11-7	-	-	0	+	+	+	cw	2-5	Beech cupules, leaves, twigs and bark, beechnuts.
Oi	OLv2	7-4	-	-	1mi,vf	++	+++	+++	cw	2-5	Beech cupules, leaves, twigs and partially
Oi	OLv3	4-3	-	-	3mi,vf,f	+++	+++	++	cw	1-2	70% of recognisable material. Brownish
Oa	OHr	3-0	10R 2.5/1	-	3mi,vf,f,m,	+	++	++	cw	2-4	Skeleton: 20% (centimetric dimension, 3-5 cm).
A1	-	0-14	2.5YR 2.5/1	3f,m sbk+abk, fr	3mi,vf,f,m; 2co	0	(+)	+	cw	14-18	Skeleton: 5% (millimetric and centimetric
A2	-	14-24	5YR 2.5/2	3f,m sbk+abk, fr	2mi,vf,f,m,co	0	(+)	(+)	aw	9-14	Skeleton: 30% (centimetric dimension, 3-5 cm).

Mount San Vicino. Parent rock: grey limestone with marl and flintstone layers; Forest management: coppice in conversion to timber forest.

Altitude: 800 m

Slope: 35°; Exposure: N; Mean annual precipitation: 825 mm; Mean annual air temperature: 10.0°C; Winter mean temperature: 1.8°C; Spring mean temperature: 8.3°C; Summer mean temperature: 19.1°C; Autumn mean temperature: 11.0°C. Drainage class: Moderately well to well drained. Vegetation: *Fagus sylvatica* (90%), *Quercus cerris, Castanea sativa;* 15% of soil cover made by: *Rubus ulmifolium,*graminae *Pteridium aquilinum, Daphne laureola,Clematis vitalba, Gallium* spp. Soil: coarse-loamy, mixed, mesic, Typic Hapludoll (Soil Survey Staff, 2014).

Oi	OLn	9-4		-	0	0	+	(+)	cw	5-7	Beech stipels, leaves, twigs and bark, beechnuts.
Oi	OLv	4-0		-	1f	0	+	+	cw	4-6	Beech stipels, leaves, twigs and partially degraded bark, beechnuts.
A	-	0-10	7.5YR 5/2	2c cr, fr	3mi,vf,f,m,co	++	(+)	(+)	cw	5-10	Skeleton: 10% (centimetric dimension, 5-10 cm).

Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2014).

Oi	OLn	7-3		-	0	0	+	(+)	cw	2-4	Beech cupules, leaves, twigs and bark.
Oi	OLv	3-0		-	1vf	+++	+	+	cw	3-4	Beech stipels, leaves, twigs, and partially degraded bark, beechnuts.
A	-	0-9		2-3f cr	3mi,vf,f,m	0	(+)	(+)	cw	6-10	Skeleton: 20% (centimetric dimension, 5-10 cm).

Altitude: 1000 m

Slope: 45°; Exposure: N; Mean annual precipitation: 825 mm; Mean annual air temperature: 9.0°C; Winter mean temperature: 0.8°C; Spring mean temperature: 7.4°C; Summer mean temperature: 18.1°C; Autumn mean temperature: 9.8°C. Drainage class: Moderately well to well drained. Vegetation: *Fagus sylvatica* (80%), *Sorbus aria, Acer* spp., *Quercus cerris;* 5% of soil cover made by: Graminae*, Crategus oxyacantha, Hedera helix, Cyclamen repandum, Prunus* spp., *Rosa canina* spp., mosses. Soil: loamy-skeletal, mixed, mesic, Lithic Haprendoll (Soil Survey Staff, 2014).

Oi	OLn	7-2	-	-	0	0	+	+	cw	4-6	Beech leaves, twigs and bark, beechnuts.
Oi	OLv	2-0	-	-	0	0	++	+	cb	1-2	Beech leaves, twigs and partially degraded bark, beechnuts. Earthworm casts.
A	-	0-7	5YR 2.5/2	3f cr,fr	3mi,vf,f,m,co	0	(+)	(+)	cw	5-7	Skeleton: 25% (centimetric dimension, 7-12 cm).

Soil: loamy-skeletal, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014).

Oi	OLn	5-3		-	0	0	+	(+)	cb	2-3	Beech leaves, twigs and bark, beechnuts.
Oi	OLv	3-0		-	0	++	++	++	cw	1-5	Beech leaves, cupules, twigs and partially degraded bark, beechnuts. Earthworm casts.
A	-	0-6	5YR 2.5/2	3f sbk+cr, fr	3mi,vf,f,m	0	(+)	(+)	cw	2-9	Skeleton: 25% (centimetric dimension, 8-14 cm).

Mount Acuto. Parent rock: white limestone with flintstone layers; Forest management: coppice in conversion to timber forest.

Altitude: 800 m

Slope: 40°; Exposure: N-NE; Mean annual precipitation: 1430 mm; Mean annual air temperature: 10.0°C; Winter mean temperature: 0.4°C; Spring mean temperature: 8°C; Summer mean temperature: 20.1°C; Autumn mean temperature: 11.5°C. Drainage class: Well drained. Vegetation: *Fagus sylvatica* (95%), *Carpinus betulus, Acer opalus, Quercus* spp.*;* 30% of soil cover made by: *Cyclamen repandum, Ruscus aculeatus, Daphne laureola, Allium Ursinum, Cardaria draba, Tamus communis, Anemone nemorosa, Luzola sylvatica, Pteriduim* spp., *Viola* spp., *Convolvulus* spp., mosses. Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2010).

Oi	OLn	7-5	-	-	0	0	+	(+)	cw	2-4	Beech cupules, leaves, twigs, bark. Few *Quercus, Acer* and *Ostrya* leaves.
Oi	OLv	5-2	-	-	0	++	+++	++	cw	3-4	Beech cupules, leaves, twigs and partially degraded bark. Few *Quercus, Acer*, *Ostrya*, and
Oa	OHr	2-0	-	-	0	+++	+++	+++	cw	2-4	Skeleton: 10-15% (centimetric dimension, 5-8 cm).
A	-	0-3	10YR 2/1	2-3f cr, vfr	2mi,vf,f	0	(+)	+	ci	1-6	Skeleton: <5% (centimetric dimension, 6-10 cm).

Soil: coarse-loamy, mixed, mesic, Typic Humudept (Soil Survey Staff, 2014).

Oi	OLn	7-5	-	-	0	0	+	(+)	cw	2-6	Beech cupules, leaves, twigs and bark. Few *Quercus, Acer* and *Ostrya* leaves.
Oi	OLv	5-2	-	-	0	++/ +++	+++	++	cw	2-5	Beech cupules, stipels, leaves, twigs and partially degraded bark. Few *Quercus, Acer* and *Ostrya*
Oa	OHr	2-0	-	-	0	+++	+++	+++	cw	2-4	
A1	-	0-3	10YR 2/1	3f cr, fr	3mi,vf,f,m,co	0	(+)	+	cw	2-3	Skeleton: 10% (centimetric dimension, 5-10 cm).
A2	-	3-9	10YR 2/2	3f cr, fr	3mi,vf,f,m,co	0	(+)	(+)	ci	5-15	Skeleton: 10% (centimetric dimension, 8-12 cm).

Altitude: 1000 m

Slope: 25°; Exposure: N-NE; Mean annual precipitation: 1430 mm; Mean annual air temperature: 9.0°C; Winter mean temperature: -0.5°C; Spring mean temperature: 7.0°C; Summer mean temperature: 19.1°C; Autumn mean temperature: 10.5°C. Drainage class: Well drained. Vegetation: *Fagus sylvatica* (100%); 50% of soil cover made by: *Daphne laureola, Hedera helix, Euphorbia amygdaloides, Anagallis arvensis, Cardamine ennaphyllos, Viola* spp., *Corydalis cava, Lamium purpureum, Stellaria* spp. Soil: coarse-loamy, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014).

Oi	OLn	5-3		-	0	0	+	(+)	cw	2-4	Beech leaves, cupules, twigs and bark, beechnuts. Earthworm casts.
Oi	OLv	3-1		-	0	++/ +++	++	++	cw	2-3	Beech leaves, twigs and partially degraded bark, beechnuts.
Oa	OHr	1-0		-		+++	+++	++	cw	1-2	
A1	-	0-11	7.5YR 3/2	3f,m sbk, fr	2mi,vf; 3f,m	0	(+)	+	cw	10-11	Skeleton: 10% (centimetric dimension, 2-12 cm).
A2	-	11-25	7.5YR 2.5/1	2f,m sbk, fr	2mi,vf,f; 3co,m	0	(+)	(+)	cw	13-15	Skeleton: 30% (centimetric dimension, 4-15 cm).

Soil: loamy-skeletal, mixed, mesic, Inceptic Haprendoll (Soil Survey Staff, 2014).

Oi	OLn	14-10		-	0	0	+	+	cw	4-7	Beech leaves, cupules, twigs, and bark, beechnuts.
Oi	OLv1	10-7		-	0	++	+++	+	cw	3-4	Skeleton: <5% (centimetric dimension, 4-8 cm). Beech leaves, twigs and partially degraded bark,
Oi	OLv2	7-4		-	0	+++	++	+	cw	3-4	Beech leaves, twigs and partially degraded bark, beechnuts.
Oa	OHr	4-0		-	2mi,vf,f	++/	++	+	cb	3-4	
A	-	0-13	7.5YR 2.5/1	3f,m cr+sbk, fr	3mi,vf,f,m; 2co	0	(+)	(+)	cw	11-16	Skeleton: 30% (centimetric dimension, 1-10 cm).

[a]horizons' designation according to Schoeneberger et al. (2012).

[b]horizons' designation according to Baize and Girard (2008).

[c]moist and crushed, according to the Munsell Soil Color Charts.

[d]1=weak, 2=moderate, 3=strong; th=thin, f=fine, m=medium, c=coarse; cr=crumb, abk=angular blocky, sbk=subangular blocky, pl=platy; fi=firm; m= moist; fr, friable; vfr, very friable; w, wet; ss, slightly

[e]0=absent, v_l=very few, 1=few, 2=plentiful, 3=abundant; mi=micro, vf=very fine, f=fine, m=medium, co=coarse.

[f]we referred to the mycelia visible at naked eyes. 0=absent, +=few, ++=plentiful, +++=abundant.

[g]we referred to the number of individuals with size from 2 to 25 mm (visible at naked eyes) present in a 40x40 cm frame, for the thickness of a given horizon, mostly made of insects, earthworms, ants,

[h]we referred to the number of individuals with size <2 mm (visible at naked eyes and with a 2x magnifying lens) present in a 10x10 cm frame, for the thickness of a given horizon, mostly made of insects, mites,

[i]a=abrupt, c=clear; b=broken, w=wavy, s=smooth, i=irregular.

through a sieve with a 1 mm mesh. Each dried A sample was deprived of the visible roots (under a magnifying lens) and sieved at 2 mm mesh.

2.3. Chemical analysis

The pH was determined potentiometrically in water (pH_{HO}) with a solid:liquid (w:v) ratios of 1:8 and 1:2.5 for O and A horizons, respectively. Total organic C (TOC) content was estimated by K-dichromate digestion, heating the suspension at 180 °C for 30 minutes (Allison, 1965), while total nitrogen (TN) was determined by dry combustion analyzer (EA-1110, Carlo Erba Instruments, Milan, Italy). To determine extractable organic carbon (C_p), we used aliquots of 2 g of not triturated samples for the OLn and OLv horizons (the representativeness of the sample was insured by considering the percentage of each organic debris composing the forest floor), and 1 g for the OH and A horizons. Each sample was shaken overnight in 30 mL of 0.1 M Na pyrophosphate solution for 16 hrs (40 revolutions min^{-1}). Thus, the mixture was transferred into plastic bottles and centrifuged (about 2400 *g*) for 15 min after the addition of 4 drops of Superfloc 0.4%. The samples were filtered by Whatman 42 filter papers. To determine the C content in the pyrophosphate extracts, 5 mL of extract from the O horizons and 12 mL of extract from the A horizons were oven-dried at 40°C in an Erlenmeyer apparatus. The dried extract was then added of 10 mL of concentrated H_2SO_4 (94% w:w) and 5 mL of 1.8 N $K_2Cr_2O_7$ solution. After 1 hour in oven at 105°C, the reaction was stopped by distillated water to 100 ml of volume. Of this solution, 5 mL were titrated with 0.1 N $FeNH_4SO_4$ solution.

2.4. Enzymatic activities

Enzymes were desorbed by heteromolecular exchange using an excess of exogenous protein (Fornasier and Margon, 2007). Enzyme activity was quantified according to Cowie et al. (2013). Briefly, 250 mg of not triturated sample from the O horizons and 250 mg of sample from the A horizons were placed in a 2-ml Eppendorf tube with glass beads and 1.2 ml of 50 mM tris–HCl solution at pH 7.0, containing 2% lysozyme as a desorbing protein. The tube was subjected to bead-beating (3 min, 30 strokes s^{-1}) using a Retsch MM400 mill, then centrifuged for 5 min at 20,000 *g*. Enzyme activity was assayed fluorometrically in microplates using 4-methyl-umbelliferyl and 7-amino-4-methyl coumarine derivatives. The activity of arylsulfatase, chitinase, glucuronidase, α-glucosidase, β-glucosidase, cellulase, xylosidase, acid phosphomonoesterase, and inositol-phosphatase was determined in 200 mM MES (morpholineptansulfonic acid) solution at pH 5.8; the activity of leucine aminopeptidase

acetate-esterase, pyrophosphatase-phosphodiesterase, and phosphodiesterase was determined in 100 mM tris–HCl solution at pH 7.5; alkaline phosphomonoesterase activity was determined in 100 mM tris–HCl solution at pH 9.0.

2.5. Statistical analysis

When the same genetic horizon was present in different morphological features (for example, OLv1 and OLv2, or A1 and A2), before each statistical treatment, a weighted mean was calculated on the basis of the respective thickness of each sub-horizon. All tests were computed with the R software (R CoreTeam, 2014).

A global view of variations and correlations of soil chemical and biochemical variables as function of horizon, latitude, and altitude were assessed by non-metric multidimensional scaling analysis (NMDS), by using the R package "vegan" with the dissimilarity matrix calculated by the Gower's distance. The fitted vectors are arrows that represent the direction of the variables and their length is proportional to the strength of the variables (Oksanen, 2015). Before the NMDS, data were standardized by subtracting the mean and dividing by the standard deviation. Analysis of variance (ANOVA) was carried out to test the effect of horizons, altitude and latitude on topsoil thickness and on chemical and biochemical properties in order to estimate significance of singular variables. To apply the ANOVA, we previously verified the normality distribution of the data and successively the equal variances. When data were not parametric with not normal distribution and heterogeneity of the variance, each numerical variable was transformed by the Box and Cox (1964) procedure using the MASS package present in R software (R CoreTeam, 2014). The improvement of the assumption to normality and homoschedasticity was verify on residuals by the Shapiro-Wilk statistic test ("stats" package) and by Levine's test, respectively ("car" package), both at 5% significance level. We considered the ANOVA test as significant when $P < 0.05$; differences between means were compared using a post-hoc Tukey's HSD test with $P < 0.05$. Only for topsoil thickness, we considered as significant both ANOVA and Tukey tests for $P < 0.1$.

3. Results

3.1. Site characteristics, and soil morphology and classification

Between the low and high altitude of all the sites, the mean annual air temperature (MAAT) differed of 1°C (Table 1). Conversely, along the small latitudinal gradient considered (1° of latitude), there was no difference in terms of MAAT, although the northernmost site (MA) was characterized by a higher summer mean temperature of 1.2 °C and a lower winter temperature of 1.7°C with respect to the southernmost site (MT). Among the sites, the

differences not directly ascribable to climatic conditions were mainly due to the slight variation in the vegetational composition of the forests, such as a greater presence of beech in MT and MA than at MV.

Soil morphology differed among profiles, altitudes and latitude (Table 1). For example, MT showed a greater sequence of both O and A horizons with respect to the other sites. Thus, the A horizons displayed different structures, with sub-angular blocks that prevails in the MT soils, crumbs in the MA soils, and an intermediate state of aggregation in the MV soils. On these structures possibly depends the soil drainage, which increased from MT to MV to MA. The soils of MT and MA showed a major biological activity represented by small macrofauna and mesofauna in the OLv and OH horizons, with a concentration of individuals that goes from plentiful to abundant. The percentage of skeleton in the A horizons was greater for MT than for MV and MA. Soils were classified as Typic Hapludolls and Lithic Haprendolls for MT, as Typic Hapludoll, Typic Humudept, Lithic Haprendoll, and Inceptic Haprendoll for MV, and as Typic Humudepts and Inceptic Haprendolls for MA.

3.2 Horizon effect

The NMDS analysis (stress = 0.067) showed relationships among all the soil attributes considered, indicating that horizons had distinct chemical and biochemical characteristics (Figure 2a). The clusters of OLn and OLv horizons were partially overlapped, indicating a slight difference between these two types of horizon for the considered properties, which much depend on the state of organic matter degradation. The OH horizons were different from the OLn horizons, and showed only a slight overlapping with the OLv cluster. However, the O horizons were frankly different from the A horizons.

The specific behavior of chemical and biochemical properties along the topsoil was analyzed singularly by ANOVA, which showed that the horizon effect was significant for all the tested parameters (Table 2). In particular, pH followed an increasing trend with depth, TOC and C/N ratio decreased from OLn to A horizons, and total N showed differences in the organic horizons and sharply decreased in the A horizon (Table 3). The C_p was the highest in the OH horizons (33.3 g kg^{-1}) and the lowest in the A horizons (17.6 g kg^{-1}). Among the enzymatic activities (Table 4), the arylsulfatase and pyrophosphatase-phosphodiesterase activities tended to increase from OLn to A horizons, with a more marked extent showed by the arylsulfatase activity. Conversely, the other enzymatic activities tended to decrease along the topsoil. One exception was the phosphodiesterase, whose activity resulted to be little affected by the degradation stage of the litter ($P \leq 0.05$) (Tables 2 and 3).

3.3 Altitude effect

On the basis of the NMDS analysis (stress = 0.069), the general pattern of chemical and biochemical properties appeared to be not affected by the different altitude (Figure 2b). In fact, all the variables obtained from the soils at 800 and 1000 m a.s.l. were substantially clustered together. However, at higher altitude, where the MAAT is 1°C lesser than that at lower altitude, the topsoil showed a slight but significant increase of thickness (Tables 1, 2, and 3). Thus, the effect of the altitude was also significant for TOC, TN, and C/N ratio (Table 2). The largest TOC and TN contents occurred in the topsoils at 1000 m altitude, where the C/N ratio was the lowest (Table 3). With regard to the enzymatic activity, only β-glucosidase and xylosidase appeared influenced by altitude (Table 2), showing greater activities at 800 m than at 1000 m (Table 4).

3.4 Latitude effect

As for altitude, 1° latitude did not seem a driving factor able to induce a generalized change in the extent of the considered variables (Figure 2c, NMDS stress = 0.067). However, ANOVA showed that topsoil thickness, pH and TN were affected by latitude (Table 2). In particular, topsoil displayed the highest thickness at MT and the lowest at MV, pH tended to decrease going north while TN followed the opposite trend (Table 3). Thus, ANOVA displayed that 9 over 14 enzymatic activities were affected by latitude (Table 2), but only for 8 of those the northernmost site (MA) significantly differed from the southernmost one (MT) (Table 4). MA showed also significant differences with respect to the intermediate site (MV) for the activity of 5 enzymes: arylsulfatase, β-glucosidase, cellulase, xylosidase and pyrophosphatase-phosphodiesterase. MA and MV had a similar value of acetate-esterase activity, higher than that detected at MT.

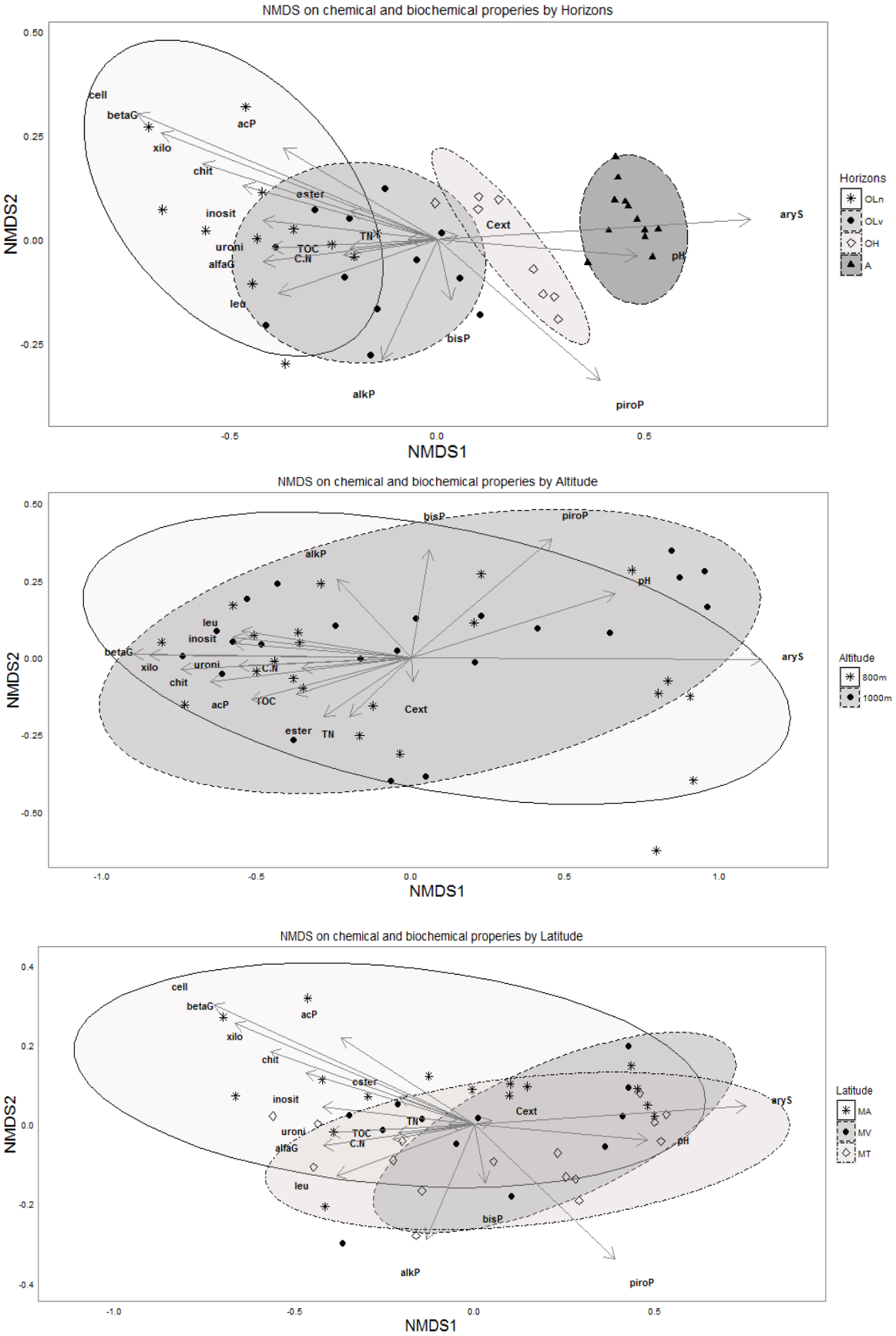

Fig. 2 - Variation of topsoil chemical and biochemical properties with the NMDS analysis acorrding to horizons (a); altitude (b) and latitude (c).

Table 2. Results of ANOVA showing the influence of horizons, latitude, and altitude on topsoil thickness and on chemical and biochemical properties for mountains of central Apennines (Italy) within 1° of latitudes.

	Horizons		Altitude		Latitude	
	F value	Significance	F value	Significance	F value	Significance
Topsoil thickness	-	-	3.75	°	4.12	°
Chemical properties						
pH	11.02	***	1.14	ns	3.30	*
TOC	277.00	***	11.49	**	1.34	ns
TN	67.83	***	22.24	***	4.73	*
C/N	87.49	***	7.14	*	1.86	ns
C_{ext}	3.09	*	0.24	ns	0.43	ns
Enzymatic activities						
AryS	50.84	***	1.54	ns	6.29	**
Chit	132.02	***	0.38	ns	5.85	**
Leu	77.95	***	0.22	ns	0.070	ns
Uroni	34.72	***	3.15	ns	0.09	ns
α-Gluco	50.15	***	0.25	ns	0.17	ns
β-Gluco	202.98	***	5.13	*	0.00	***
Cell	62.78	***	0.97	ns	6.23	**
Xilo	228.94	***	6.72	*	12.64	***
AcPME	30.21	***	1.87	ns	1.75	ns
BisP	3.15	*	3.00	ns	5.60	**
PyroP	5.20	**	0.85	ns	15.83	***
AlkPME	16.44	***	3.39	ns	4.26	*
Inosit	97.61	***	2.48	ns	1.29	ns
Ester	23.52	***	3.98	ns	9.79	***

Level of significance: ns = not significant, ° = $P < 0.1$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

AryS=arylsulfatase; Chit=chitinase; Uroni=glucuronidase; α-Gluco=α-glucosidase; β-Gluco=β-glucosidase; Cell=cellulase; Xilo=xylosidase; AcPME=acid phosphomonoesterase; BisP=phosphodiesterase; PiroP=pyrophosphatase-phosphodiesterase; AlkPME=alkaline phosphomonoesterase; Inosit=inositol-phosphatase; Ester=acetate-esterase.

Table 3. Topsoil thickness, pH, total organic carbon (TOC), Na-pyrophosphate extractable organic carbon (C_p), total nitrogen (TN), and carbon/nitrogen ratio (C/N) of the topsoil at a micro- (horizon), meso- (altitude), and macro- (latitude) scale for mountains of central Apennines (Italy) within 1° of latitudes. The numbers in parentheses are the standard deviations. For each column and variable (horizons, altitude, and latitude), mean values with different letters significantly differ for $P < 0.05$ ($P < 0.1$ in the case of horizons' thickness) for the Tukey's post-hoc test.

	Topsoil thickness	pH	TOC	C_p	TN	C/N
	cm		g kg^{-1}			
Horizons						
OLn	-	6.18^{c}	506.2^{a}	25.4^{ab}	14.5^{b}	36.0^{a}
		(0.23)	(64.2)	(14.65)	(3.19)	(6.4)
OLv	-	6.51^{bc}	496.4^{a}	29.7^{ab}	15.9^{ab}	31.3^{a}
		(0.33)	(58.6)	(13.46)	(2.18)	(3.7)
OH	-	6.91^{ab}	409.1^{b}	33.3^{a}	17.8^{a}	23.4^{b}
		(0.50)	(63.1)	(6.16)	(3.8)	(3.7)
A	-	7.18^{a}	79.3^{c}	17.6^{b}	6.0^{c}	14.0^{c}
		(0.70)	(36.0)	(5.39)	(3.0)	(3.2)
Altitude						
800 m	17.2^{b}	6.60^{a}	344.1^{b}	25.8^{a}	11.6^{b}	27.8^{a}
	(4.4)	(0.6)	(187.1)	(9.72)	(5.4)	(9.7)
1000 m	28.0^{a}	6.75^{a}	394.8^{a}	25.9^{a}	14.7^{a}	25.1^{b}
	(13.8)	(0.6)	(195.5)	(14.45)	(5.0)	(9.9)
Latitude						
MT	32.0^{a}	6.92^{a}	352.9^{a}	26.1^{a}	11.9^{b}	28.3^{a}
	(13.0)	(0.66)	(181.5)	(10.9)	(4.2)	(11.5)
MV	15.0^{b}	6.57^{ab}	366.65^{a}	25.0^{a}	13.0^{ab}	26.1^{a}
	(3.4)	(0.45)	(198.9)	(12.7)	(4.87)	(9.2)
MA	20.8^{ab}	6.52^{b}	388.2^{a}	26.3^{a}	14.6^{a}	24.8^{a}
	(9.4)	(0.59)	(204.5)	(13.7)	(6.7)	(8.5)

MT=Mount Terminillo; MV=Mount San Vicino; MA=Mount Acuto.

Table 4. Soil enzymatic activities of the topsoil at the micro- (horizon), meso- (altitude), and macro- (latitude) scale for mountains of central Apennines (Italy) within 1° of latitudes. The numbers in parentheses are the standard deviations. For each column and variable (horizons, altitude, and latitude), mean values with different letters significantly differ for $P < 0.05$ ($P < 0.1$ in the case of horizons' thickness) for the Tukey's post-hoc test.

	AryS	Chit	Leu	Uroni	α-Gluco	β-Gluco	Cell	Xilo	AcPME	BisP	PyroP	AlkPME	Inosit	Ester
	nanomoles of 4-methylumbelliferone (7-amino-4-methyl coumarine) • g^{-1} dry soil • hour^{-1}													
Horizons														
OLn	8.0^{c}	216.1^{a}	372.5^{a}	5.2^{a}	10.6^{a}	621.7^{a}	97.3^{a}	63.4^{a}	434.1^{a}	51.5^{a}	19.4^{b}	389.7^{a}	36.0^{a}	3742.6^{a}
	(4.6)	(68.3)	(129.4)	(1.6)	(2.5)	(322.7)	(67.5)	(26.0)	(305.3)	(14.0)	(13.1)	(173.6)	(10.6)	(794.3)
OLv	13.0^{c}	120.89^{b}	376.9^{a}	3.9^{a}	7.9^{a}	215.8^{b}	31.2^{b}	29.6^{b}	283.5^{a}	45.6^{ab}	35.2^{ab}	377.7^{a}	24.8^{b}	3129.4^{a}
	(8.9)	(48.0)	234.8	(2.3)	(3.8)	(172.4)	(23.2)	(13.3)	(106.8)	(11.15)	(24.0)	(135.4)	(9.2)	(847.0)
OH	28.1^{b}	48.4^{c}	98.7^{b}	1.9^{b}	2.3^{b}	33.9^{c}	3.8^{c}	6.8^{c}	159.3^{b}	37.2^{b}	45.2^{a}	331.2^{a}	9.1^{c}	1680.8^{b}
	(21.2)	(24.9)	(47.8)	(1.6)	(1.0)	(26.4)	(2.7)	(4.5)	(56.6)	(13.1)	(34.8)	(107.3)	(2.8)	(894.0)
A	65.9^{a}	5.1^{d}	24.8^{c}	0.7^{c}	0.7^{c}	4.4^{d}	0.3^{c}	1.0^{d}	92.0^{c}	41.5^{ab}	33.1^{ab}	158.1^{b}	4.7^{d}	1498.9^{b}
	(11.4)	(4.5)	(12.4)	(0.2)	(0.4)	(1.7)	(0.1)	(0.6)	(34.9)	(11.32)	(16.8)	(50.1)	(1.2)	(838.0)
Altitude														
800 m	28.2^{a}	102.6^{a}	228.7^{a}	3.2^{a}	5.4^{a}	255.9^{a}	36.9^{a}	28.6^{a}	282.5^{a}	41.6^{a}	30.8^{a}	283.8^{a}	20.8^{a}	2721.5^{a}
	(28.2)	(90.5)	(180.3)	(2.1)	(4.3)	(337.5)	(53.9)	(30.6)	(277.3)	(11.7)	(25.5)	(139.3)	(15.5)	(1056.9)
1000 m	29.4^{a}	101.6^{a}	229.4^{a}	3.8^{a}	6.7^{a}	215.9^{b}	42.1^{a}	25.1^{b}	217.1^{a}	47.5^{a}	33.5^{a}	341.4^{a}	18.3^{a}	2455.7^{a}
	(25.5)	(89.9)	(246.1)	(2.7)	(5.3)	(295.1)	(58.6)	(28.6)	(121.7)	(13.8)	(21.3)	(170.3)	(14.2)	(1451.5)
Latitude														
MT	34.0^{a}	91.7^{a}	263.6^{a}	2.9^{a}	5.9^{a}	194.0^{b}	32.5^{b}	20.7^{b}	199.5^{a}	50.1^{a}	45.4^{a}	361.4^{a}	17.8^{a}	2035.4^{b}
	(28.6)	(84.8)	(273.5)	(2.3)	(5.1)	(273.8)	(40.9)	(25.2)	(107.6)	(8.3)	(26.5)	(138.4)	(13.4)	(1244.0)
MV	32.9^{a}	79.8^{a}	189.5^{a}	3.3^{a}	5.4^{a}	138.7^{b}	20.9^{b}	23.5^{b}	225.7^{a}	46.0^{b}	35.4^{a}	328.7^{ab}	19.8^{a}	2900.6^{a}
	(28.5)	(66.5)	(134.4)	(2.0)	(4.2)	(145.2)	(23.2)	(20.0)	(107.8)	(16.0)	(22.1)	(205.9)	(14.1)	(824.5)
MA	20.6^{b}	129.2^{a}	224.3^{a}	4.1^{a}	6.5^{a}	350.4^{a}	60.0^{a}	35.6^{a}	318.2^{a}	37.9^{b}	16.5^{b}	251.7^{b}	21.0^{a}	2907.7^{a}
	(22.3)	(115.8)	(199.2)	(2.7)	(5.1)	(410.7)	(76.9)	(37.4)	(321.3)	(12.1)	(6.0)	(115.2)	(17.0)	(1416.5)

AryS=arylsulfatase; Chit=chitinase; Leu=leucine-aminopeptidase; Uroni=glucuronidase; α-Gluco=alpha-glucosidase; β-Gluco=beta-glucosidase; Cell=cellulase; Xilo=xylosidase; AcPME=acid phosphomonoesterase; BisP=phosphodiesterase; PiroP=pyrophosphatase-phosphodiesterase; AlkPME=alkaline phosphomonoesterase; Inosit=inositol-phosphatase; Ester=acetate-esterase.

4. Discussion

4.1 Horizon effect

The degradation processes of the shed vegetal debris form a stratified litter floor with a gradient of chemical characteristics and enzyme activities that goes from the rather undecomposed OLn horizon to the mineral A horizon (e.g., Baldrian, 2014; Šnajdr et al., 2008). This gradient was evident in the studied topsoils, where most of the observed morphological, chemical and biochemical parameters differed within the O horizons and between the O and A horizons. Further, the NMDS analysis conducted on the entire pattern of variables stressed that the differences in chemical and biochemical properties of the topsoil were peculiar for each horizon according to the degradation processes.

The higher content of TOC in the upper OLn horizons was due to a scarce degradation level of the fresh residues (Wittmann et al., 2004), although TOC includes organic matter that spans from fresh organic debris to low molecular weight organics derived from the activity of micro- and mesofauna, and microrganisms (Schröder, 2008). Thus, it was expected that a decrease of TOC content would correspond to an increase of the extractable carbon content, even if the trend of this latter was not statistically significant for the O horizons. However, the contribution of extractable carbon to TOC increased from OLn (5.0 %) to OLv (6.0 %), to OH (8.1 %) and, much more, to A (22.2 %) horizons. Probably because of this, the activity of the enzymes devoted to the first steps of organic matter degradation (chitinase, leucine-aminopeptidase, glucuronidase, alpha-glucosidase, beta-glucosidase, cellulase, xylosidase, acid phosphomonoesterase, inositol-phosphatase, acetate-esterase) gradually decreased with depth, while the activity of the enzymes involved in advanced stages of organic matter decay (arylsulfatase, pyrophosphatase-phosphodiesterase) increased. Our results partially agreed with those of Herold et al. (2014), who found that most of the enzymatic activities decreased with depth because of the reduced fresh C input into the deeper horizons. As a support of our findings, the decreasing trend with depth of the C/N ratio indicated the presence of a relatively more degradated (humified) organic matter in the deeper horizons. According to the NMDS results, the general direction of TOC and C/N ratio vectors was similar to that of α-glucosidase, β-glucosidase, cellulase, xylosidase and glucuronidase activities. These enzymes are involved in the first steps of organic matter decay, being them related with the degradation of cellulose, hemicellulose, pectins and lignin, which are the main components of leaves and wood. In addition to these enzymes, also chitinase and leucine-aminopeptidase activity was in the same general direction of TOC and C/N ratio. Chitinase is able to hydrolyze chitin, an important component of fungi cell wall and of the exoskeleton of arthropods (Kjøller and Struwe, 2002), while leucine-aminopeptidase is involved in the degradation of proteins (Burley et al., 1991).

Consequently, due to the input of fresh vegetal debris and the presence of macro and mesofauna, the activity of the abovementioned enzymes was the highest in the OLn horizons, and decreased in the A horizons. Among the enzymes responsible for P mineralization, also inositol-phosphatase and alkaline phosphomonoesterase were more active in the O horizons. As inositol-phosphates are components of plant tissues (Turner et al., 2002b), it is possible that the accumulation of vegetal debris in the OLn horizons have led to a major production and activity of the enzyme involved in its degradation, to decrease along the topsoil. The activity of phosphodiesterase, apparently lower than that of acid and alkaline phosphomonoesterase, was attributed to the release of P monoesters from P diesters, which stimulate the phosphomonoesterase activity (Hou et al., 2015; Nannipieri et al., 2011).

In the NMDS graph, the pH and the arylsulfatase activity showed an opposite trend with respect to the majority of the chemical and biochemical characteristics considered. Arylsulfatase is a sulfohydrolase that plays a central role in the turnover of S in soil, as it can catalyze the hydrolysis of organic sulfate esters so producing S forms that are available for plants (Houghton and Rose, 1976; McGill and Cole, 1981; Tabatabai, 1994). This enzyme is produced mainly by fungi and bacteria, but plants and animals are able to produce it too (Fitzgerard, 1978). Arylsulfatase was one of the first sulfatase to be detected in soil (Tabatabai, 1994) but, as far as we know, there is a lack of information about the arylsulfatase activities in the organic horizons of deciduous forest soils. However, similarly to the strong correlation with pH values found in mineral horizons (Deng and Tabatabai, 1997), arylsulfatase activity showed a strong correlation with pH (R^2=0.949) also for the organic horizons (OLn, OLv, and OH) of the studied soils (Fig. S1a of Supplementary material). The arylsulfatase activity was the highest in the A horizons, where the mean pH value (7.18) was out of the pH optimum (5.5-6.2) for its activity (Tabatabai and Bremner, 1970). Several studies reported that the activity of arylsulfatase is also correlated with the soil organic carbon content (e.g., Dick et al., 1988; Tabatabai, 1994; Taylor et al., 2002). In our organic horizons, arylsulfatase activity was inversely correlated with TOC (R^2=0.978), and positively correlated with extractable organic carbon (R^2=0.893) (Fig. S1b and S1c of Supplementary materials, respectively), indicating that this enzyme increased its activity when organic matter had reached advanced decay stages. Prietzel (2001) reported that, for different soil types and litter qualities, arylsulfatase activity increased from OF to OH horizons and decreased in the first mineral layer (0-10 cm), showing that the higher activity was in the organic horizons with a high humus content and ester sulfate concentrations. Indeed, the release of S is due to microorganisms that mineralize S-bearing compounds bound to the humic fraction by using the organic carbon as source of energy (Casella, 1993). In our study, the highest arylsulfatase activity was detected in the A horizons, where highest

was the degradation degree of the organic matter and, because of this, there was a more suitable environment for the microbial community responsible for the breakdown of the S-bearing compound than in the O horizons.

Pyrophosphatase activity is devoted to hydrolyzing the pyrophosphate, a byproduct of nucleic acids, carbohydrates, proteins and fatty acids degradation, in orthophospate (Hernández-Domíguez et al., 2012). Pyrophosphatase is ubiquitous in soil since it is present in fungal, animal and plant tissues, and microorganisms (Reitzel, 2014). Tabatabai and Dick (1979) reported that pyrophosphatase activity in mineral soils is high in the upper horizons and decreases with depth due to the significant drop of organic C. However, Parent and MacKenzie (1985) reported that pyrophospatases are more easily extractable from the fibric horizons (corresponding to our OLn and OLv horizons) than from horizons with higher content of humic substances as the complexes between organic colloids and enzymes are resistant to extraction. In our case, as pyrophosphatase activity increased from OLn to the horizons below, its trend was ascribed to the degradation level of the organic matter rather than to the amount of TOC, which showed no correlation with pyrophosphatase activity (R^2=0.031, Fig. 2a of Supplementary materials). As a partial demonstration of this, pyrophosphatase activity was, instead, well correlated with pH in the O horizons (R^2=0.966) and extractable carbon (R^2=0.994) (Fig. 2b and 2c of Supplementary materials, respectively,). Evidently, notwithstanding the formation of colloid-enzyme complexes, the organic matter decaying occurring in the O horizons (with consequent availability of nucleic acids, carbohydrates, proteins and fatty acids), is able to supply pyrophosphate molecules so to foster the production of pyrophosphatases.

4.3 Altitude effect

The investigated sites at 800 and 1000 m a.s.l. (meso-scale level), with their temperature difference of 1°C, showed similar soils (Figure 2b), with differences only for few of the considered parameters. In particular, differences were observed for the thickness of the topsoil, and the TOC and TN contents, that were higher at 1000 than at 800 m. Similar results were reported by De Feudis et al. (2016) for the same environments. The higher thickness of the topsoil and content of TOC and TN at the highest altitude were attributed to the colder temperature of the sites that, by reducing microbial activity, has allowed a greater accumulation of organic matter (Garten and Hanson, 2006; Tsui et al., 2013). Among the enzymes, only β-glucosidase and xilosidase activities appeared to be affected by the altitude as they decreased their activity with the upward shifting. In particular, β-glucosidase activity is known to be sensitive to temperature (German et al., 2012), and it is considered an important parameter for soil quality monitoring due to its role in the turnover of soil organic matter (Turner et al., 2002a). The rising of temperature of 1°C, in a perspective of

temperature changes, might lead to an increase of β-glucosidase and xilosidase activities at the higher altitudes, with a consequent more intense degradation of cellulose and hemicelluloses, and a re-arrangement of the forest floor. In this way, at least in the areas around 1000 m a.s.l., we could expect a reduction of the thickness of the organic and organo-mineral horizons, and a minor content of organic C and N. Since litter degradation is considered as a direct source of CO_2 to the atmosphere (Coqteaux et al., 1995), the consequence of its enhanced decomposition rate could affect the equilibria regulating the concentration of atmospheric CO_2.

4.4 *Latitude effect*

As we wanted to investigate on the changes of topsoil chemical and biological characteristics on a macro-scale level (latitude), we selected sites with similar geology, exposure, topography, MAAT, soil and vegetation cover; because of this, the three sites covered a latitudinal gradient as small as 1°. The similarity of the environmental conditions reflected on the general soil chemical and biological properties, as the NMDS analysis highlighted that MA, MV and MZ soils were comparable (Figure 2c). Because of this, no one of the environmental factors taken into consideration can explain the differences among the sites in terms of pH and TN, and of arylsulfatase, β-glucosidase, cellulase, xylosidase, phosphodiesterase, pyrophosphatase-phosphodiesterase, alkaline phosphomonoesterase, and acetate-esterase activities. As MAAT was the same at the three latitudes, to explain the changes of these soil chemical and biochemical properties along the latitudinal gradient, we took into consideration the mean seasonal air temperature. From the analyses of the mean monthly temperature, we observed that the northernmost site (MA) was characterized by the lowest winter value and the highest summer value (Table 5).

Table 5. Mean summer and winter air temperature, and summer-winter thermal excursion (ΔT) for the areas at 800 and 1000 m of altitude, and mean of the thermal excursions at 800 and 1000 m of altitude (MΔT) for mountains of central Apennines (Italy) within 1° of latitudes.

	800 m a.s.l.			1000 m a.s.l.			MΔT
	Summer	Winter	ΔT	Summer	Winter	ΔT	
	°C						
Mount Terminillo	18.9	2.1	16.8	17.7	1.3	16.4	16.6
Mount SanVicino	19.1	1.8	17.3	18.1	0.8	17.3	17.3
Mount Acuto	20.1	0.4	19.7	19.0	-0.5	19.5	19.6

In contrast, the southernmost site (MT) had milder winters and cooler summers than the other sites (Table 5). . Consequently, while along the south-to-north gradient MAAT was constant, the summer-winter temperature excursion increased of about 3°C at both 800 and 1000 m a.s.l.. Because of this, and in absence, as far as we know, of information about the effect of thermal

excursion on the changes of soil properties along latitudinal transects, we ascribed to this parameter the observed chemical and biochemical changes. Figure 3 shows that correlations between the eight enzymatic activities that differed among sites and the mean summer-winter thermal excursions for each site are all significant, with R^2 values that vary from 0.50 to 1.

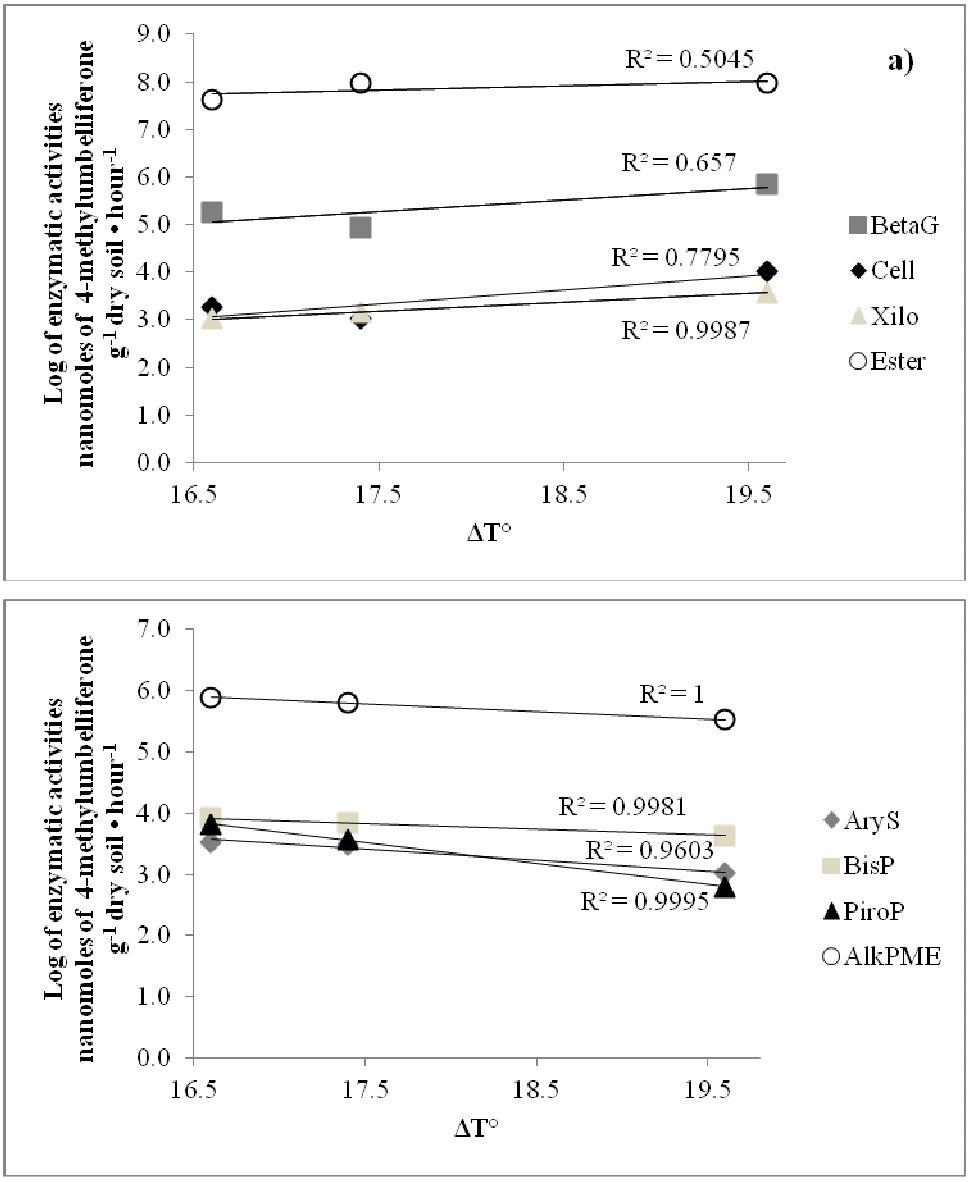

Fig. 3 - Correlation of enzymatic activities with thermal averaged range between winter and summer.

In particular, enzymes involved in the C-cycle such as β-glucosidase, cellulase, xylosidase, and acetate-esterase increased their activities from south to north (Figure 3a), while arylsulfatase and the enzymes involved in P-cycle (phosphodiesterase, pyrophosphatase-phosphodiesterase, and alkaline phosphomonoesterase) followed the opposite trend (Figure 3b). To explain this circumstance, we must take into consideration that the temperature change may not to be directly

able to modify the production of enzymes, but it may act indirectly by inducing modifications on the amount of available substrate, and on the microbial nutrient demand and activity (Sweden et al., 2016). Also, a wider thermal excursion corresponds to more intense stress conditions for plant and microrganisms. In these conditions these latter are forced to increase their activity, also by increasing production of enzymes devoted to the breakdown of the organics for energetic purposes. Hence, we speculated that the increase of thermal excursion amplitude stimulated the production (activity) of the C-cycle related enzymes (Figure 3a). As a support of this hypothesis, soil pH decreased and TN increased along the same gradient. The pH decreasing was probably due to the higher production of CO_2 promoted by microorganism activity, while the TN increased because of the differential release of C over N during the use of organic matter for energy production. For the inverse behavior of arylsulfatase and the P-cycle related enzymes, which decreased with increasing thermal excursion (Fig. 3b), we suspected that drainage could have affected this situation. Indeed, as drainage class intensity increased from south to north, the topsoil of the northernmost sites would undergo greater leaching than at southernmost ones, and this could have favoured the loss of the intermediate molecules produced during the organics breakdown, so limiting the arylsulfatase and the P-cycle related enzymes activity.

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Supplementary material

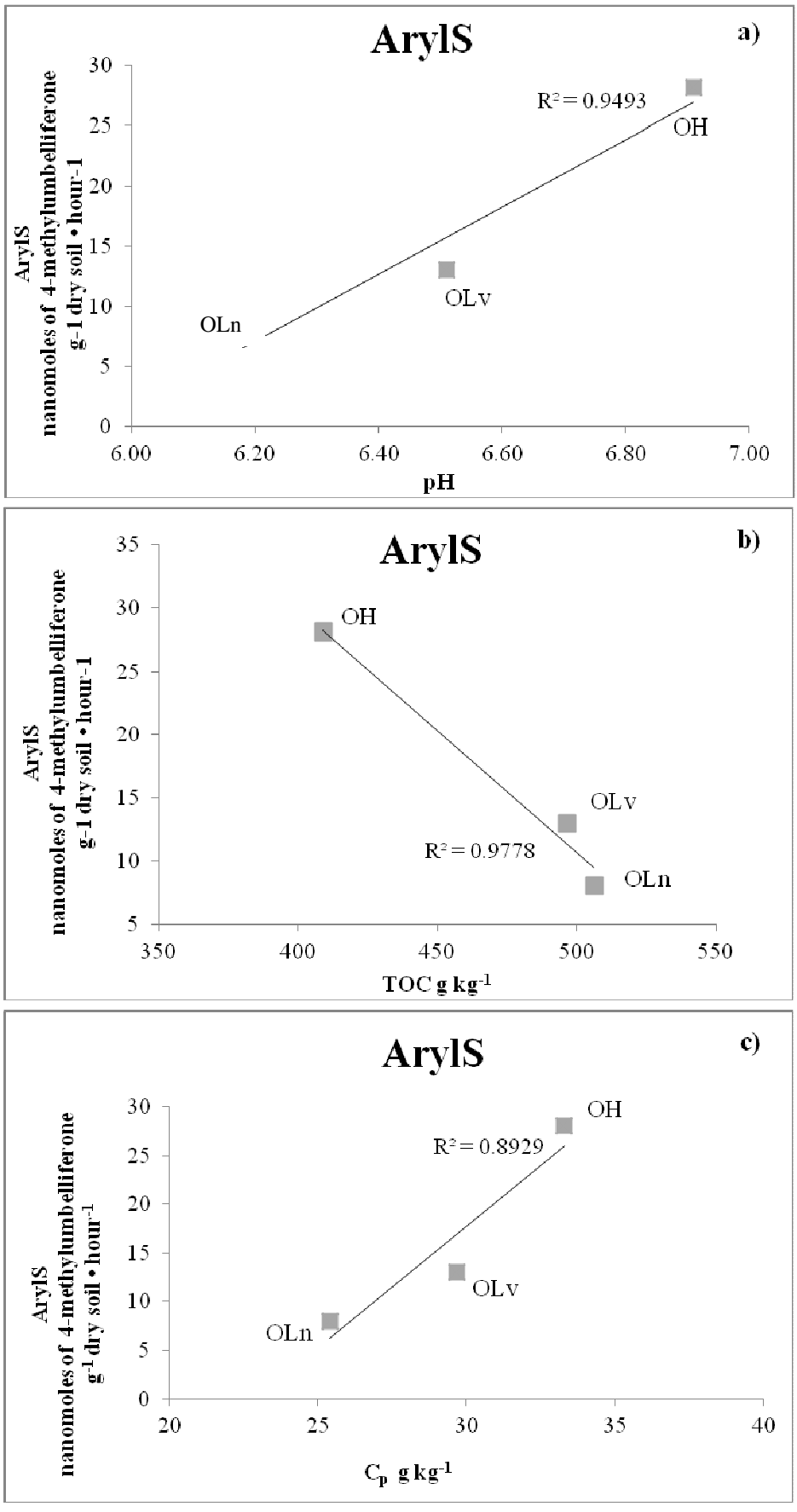
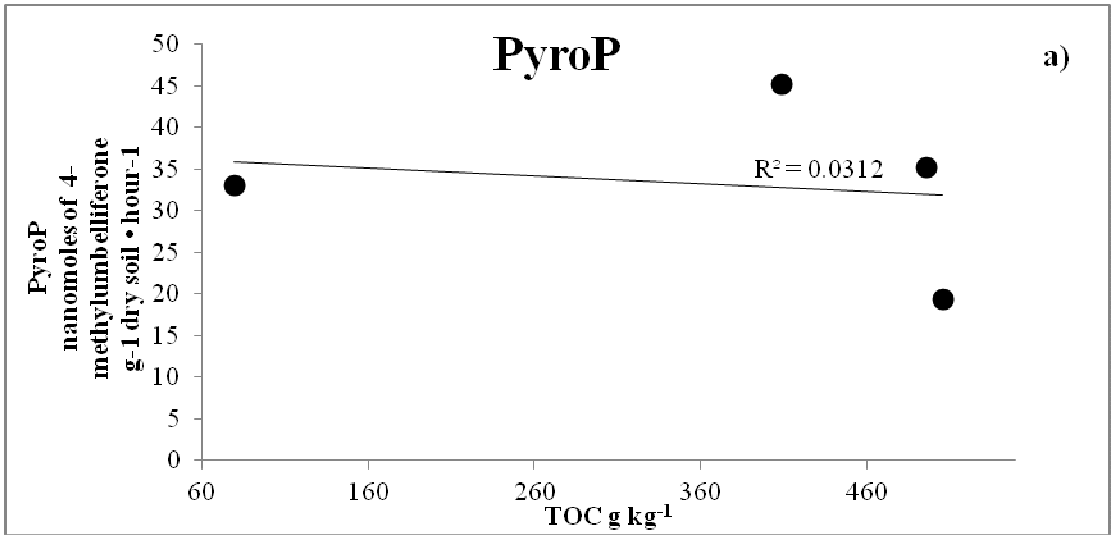
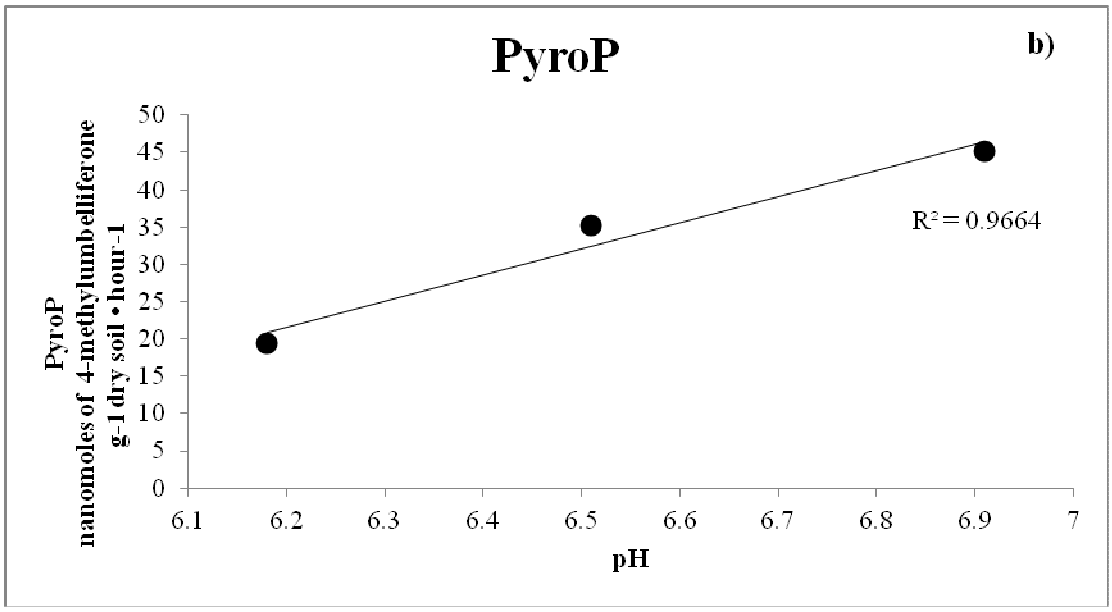


Fig S1 - Linear correlation of arylsulphatase activity with pH (a), TOC (b), and pyrophosphate extractable organic carbon (C_p) (c).





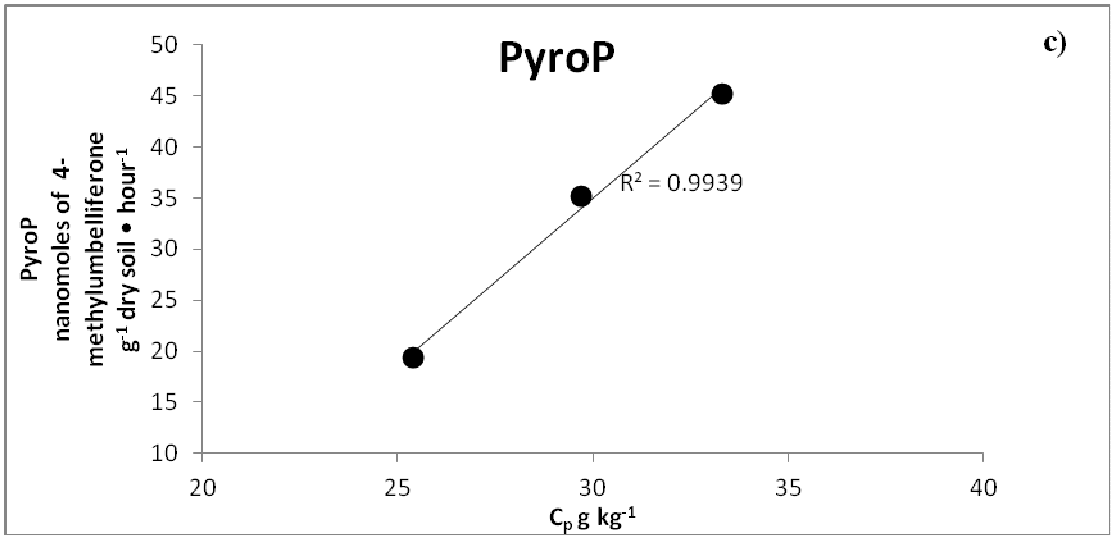


Fig S2 - Linerar correlation of pyrophosphatase-phosphodiesterase activity with TOC (a), pH (b), and pyrophosphate extractable organic carbon (C_p) (c).

3 General discussion

In this doctoral research several aspects concerning plant-soil system of beech forest environments from central Apennines (Italy) were evaluated, with particular focus on the effect of temperature changes. Soil profiles were opened according to the reference procedure and samples were collected from the first organic horizons, indicated as organic litter (OL), to the parent material (C horizon). Three sites where selected along a 1° latitudinal transect along the Apennines chain, with no difference in mean annual air temperature but with differences of summer-winter thermal excursion. For each site, soil profiles were opened at two different altitudes (800 and 1000 m) with a difference of 1°C in the mean annual air temperature between them. For each profile, specific environmental parameters were maintained in order to reduce the heterogeneity typical of soils from natural environments and allow comparing results. All the soils studied developed from limestone and faced the north-facing slope of coppice beech forests or coppice in conversion to timber forests.

Significant changes occurred along the altitudinal-latitudinal transect for all the aspect considered. Horizons by horizons comparison between rhizosphere and bulk properties confirmed the rhizosphere as an environment enriched in 1) total organic carbon (TOC), differentiated also in its more labile form (WEOM, the water extractable organic matter), with a greater concentration of tannins and condensed aromatic compounds, and 2) available P, since plant roots are able to mobilize P by releasing enzymes and exudating low molecular weight organic compounds. Results also showed that at 1000 m differences between rhizosphere and bulk soil were the highest, and TOC and total nitrogen were in greater concentration, thus confirming the existence of both a rhizosphere and an altitudinal effect. An accumulation of TOC and total nitrogen at 1000 m for both mineral and organic horizons was related to the lower temperature of the sites at this altitude. Here, also a greater thickness of organic horizons was recorded. Although the plant biomass production was possibly reduced because of the lower temperature, the increased thickness of the litter was evidently counterbalanced by the lowered microbial activities responsible for the degradation of organics. As a support of this, the enzymatic activities of β-glucosidase and xylosidase, used as surrogates of microbial communities, resulted more effective at 800 m, indicating a more accelerated organic matter cycling at the lower altitude, as seen for both the soil fractions of the mineral horizons. The analysis of the chemical structure of organic matter of rhizosphere and bulk soil remarked the strong influence of the decomposition of the topsoil (organic horizons and the underlying A horizons). The higher activities of related C-cycle enzymes in the OL horizons supported the hypothesis of a major incorporation of cellulose and hemicellulose derived substances

in the underlying mineral soil, with no distinction between rhizosphere and bulk soil. The general overview indicates that temperature may influence quantity and quality of litter in European beech forest soils, and may have consequences on the organic matter stocked in the mineral horizons. However, latitude appeared to have scarce effect on mineral horizons, while in the organic ones we highlighted an unexpected correlation between enzymatic activities and seasonal thermal excursion. The positive response of a pattern of enzymes in the organic rather than in the mineral horizons can be explained with the higher climatic rigor experienced by microbial communities of the superficial organic horizons with respect to those of the subsoil. Notwithstanding the significant findings obtained, future research is needed to further investigate the complex relationships between plant and soil, focusing on the key role of forest floor and organic layers as a direct link between the two systems. The new perspective of improving methodologies that allow obtaining reliable results from the analysis of organic and mineral horizons by saving time and sample amount, is a challenge launched during this doctoral research. This arguments is far to be exhaustively explored, hence there is the hope it will be followed by future studies aimed to save money, chemicals and time for the analysis of soil organic horizons.

Future articles and paper are improving to be published.

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