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BIOPHYSICAL CHARACTERIZATION OF SOIL AGGREGATE FRACTIONS IN DIFFERENT AGROECOSYSTEMS

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Abstract

Aggregates contribute to determining the structure of the soil and form the physical space and the habitat in which microorganisms live and play their role by regulating soil functioning. Consequently, the study of the biophysical properties of aggregates can be an effective tool for assessing what influence soil management has on its functionality, and especially on carbon sequestration.

Interdisciplinary research on the biophysical properties of aggregates therefore needs to be carried out in order to assess the effect of management on the biophysical properties of different aggregate size classes. In this study we investigated the biophysical parameters of macroaggregates (4-1 mm) mesoaggregates (1-0.25 mm) and microaggregates (<250 μm) in soils under an alfalfa crop and oak wood (representative of a mountain agroecosystem), and under three walnut sites (representative of plain agroecosystem) characterized by differing urea distribution (one site was fertirrigated with 90 kg liquid urea/ha^{-1}, one site received 90 kg granular urea/ha^{-1}, one site acted as the control without urea addition). We assumed that different aggregate classes (different microhabitats) have specific biophysical properties and the spatial relationship between organic matter and pores should be different in aggregate classes, regulating soil carbon sequestration function.

Our biophysical characterization showed that the aggregate classes investigated were easily distinguishable microhabitats. The soil management effects depended on aggregate size. Soil organic matter input and N fertilization affected the soil organic matter availability for microorganisms in macroaggregates. The aggregation process, by contrast, seemed more relevant for the C dynamics in meso- and microaggregates, thus in aggregates <1 mm. Indeed, thin aggregate sections confirmed that mesoaggregates were microhabitats in which a great accumulation of organic matter occurred as stable and transformed amorphous forms, as a result of aggregate genesis.

1. INTRODUCTION

1.1 Soil functions and C sequestration

Soil is a very complex system. It may be described as a multicomponent and multifunctional system with definable operating limits and a characteristic spatial configuration. In the soil system, most internal functions interact in a variety of ways across a range of spatial and temporal scales. Soil plays a role in sustaining the wellbeing of humans and of society (Bouma, 2014), and in supporting ecosystem services through soil functions (Adhikari and Hartemink, 2016). Soil functions are closely related to soil quality, which is defined by Karlen et al., (1997) as "the capacity of a specific kind of soil to function within natural or managed ecosystem boundaries...", emphasizing the multi-functionality of soils and their chemical, physical and biological properties. The soil, therefore, performs different key functions on an environmental level, but even on a social and economic level. Seven soil functions have been defined as follows (EC, 2006):

(i) Production of foodstuff and other biomasses, both agricultural and forestry-based. It was estimated by FAO that approximately 90% of the global agro-food production (in terms of calories) is destined for human consumption, and derived from productions based on the soil system.

(ii) Accumulation, filter and transformation of nutrients, water and other substances. This particular soil function clearly works as an "open" system. One has but to think of the influence that soil has on the surface and groundwater cycle. The soil receives a number of deposits both solid and liquid, and interacts with these deposits through mechanical filtration, physico-chemical absorption, precipitation, decomposition and mineralization. All of these interactions have an effect on groundwater quality. Many of these reactions also contribute to global environmental changes, particularly in terms of greenhouse emissions into the atmosphere.

(iii) The biodiversity pool, in terms of habitat, species and genes. Soil contains more species than any other environmental compartment.

(iv) A physical substrate for human activities, for example industrial, socio-economical and recreational activities.

(v) A source of raw materials (i.e. gravel, sand, minerals and coal).

(vi) A sink for C. Soil organic C content is about three times higher than we find in the surface biomass and about twice the content we can find in the atmosphere.

(vii) Archives of our geological and archeological heritage. Soil constitutes an essential part of the world we live in; it hides and protects archeological treasures that are fundamental to understanding our history and the history of our planet.

Atmospheric concentration of carbon dioxide CO_2 has increased considerably during the last century, because of anthropogenic emissions resulting from the use of fossil fuels and changes in the use of soil (IPCC, 2001). In this context, the soil performs its function as a C sink by the accumulating soil organic matter, and consequently sequestrating atmospheric CO_2 as a carbon pool that has long-term stability. Awareness of the potential use of soil as a carbon sink (and therefore, as a possible means of reducing of CO_2 and greenhouse gases) has increased interest in the C cycle and the control mechanisms behind the seizure of this element in the soil. Different ecosystems have different mechanisms and carbon sequestration rates (Berg and McCougherty, 2008; IPCC, 2000). Accumulation and turnover rates of C in the soil are not easy to evaluate (Torn et al. 1997; Homann et al., 1998) and the first step is always an inventory of soil content and organic carbon stock (Houghton et al., 2012; Scharlemann et al., 2014; Oertel et al., 2016), in order to understand how carbon flows through the various compartments.

The biogeochemical cycle of carbon affects all terrestrial ecosystems and concerns the absorption and transformation of carbon in terrestrial (biota and soil), atmospheric and water compartments. The quantity of soil organic carbon is defined as the balance between carbon input and carbon loss. Organic carbon enters the soil mainly through the input of plant residual material and dead animals, but also through the exudates of the root and the external addition of organic material, such as amendments or organic fertilizer (i.e. manure, compost, etc.). CO_2 flow, produced by the soil and leading to C loss, originates from various sources, the two most important being (1) heterotrophic respiration, attributable to soil microorganisms that decompose organic matter, and (2) autotrophic respiration, attributable to plant roots (Flattery et al., 2018). On a global level, soil respiration is one of the biggest CO_2 flows producing 50-80 Pg C/year (Raich et al., 2002). Soil respiration rates vary spatially and temporally under the influence of various environmental factors, such as temperature, humidity conditions, precipitation, disturbances (e.g. fire), types of vegetation and its density, and root activity (Schlesinger, 1977; Oertel et al., 2016). Vegetation controls the storage of carbon in two ways: the net primary productivity of vegetation determines the rate of C inputs to soil organic matter, and secondly, vegetation also controls the decomposition of organic matter added to the soil and hence the soil structure (Melillo et al., 1982). After all the diversity of the soil structure, the density of bacterial and fungal colonies and the density of the roots are correlated with a heterogeneous flow of CO_2 from the soil (Högberg et al., 2005). It is therefore evident that a global scale phenomenon occurs on a at microhabitat soil scale, driven by the environmental conditions.

1.2 *Soil management and its consequences for C storage*

Soil organic matter accumulation depends on the balance between carbon input and carbon loss, as described above; suitable soil management should increase organic residue return to the soil and limit carbon depletion, greatly improving soil organic matter (SOM) stabilization. Several researchers (Stevenson, 1994; Christensen, 1996; Six et al., 2002) have proposed three main mechanisms of SOM stabilization: (1) physical protection, (2) stabilization by organo-mineral bonding, and (3) biochemical stabilization. Basically, these mechanisms involve the accessibility of SOM to microbes and enzymes, the interactions between organic and mineral compounds, and the chemical resistance of organic molecules to microbial attack, respectively.

Soil organic matter can be physically (1) protected against microbial decomposition by soil aggregation. Several studies have unfolded the relationship between aggregate dynamics and associated soil organic matter dynamics (Jastrow, 1996; Six et al., 1998 and 2000). According to Elliott and Coleman (1988) aggregates protect SOM by forming physical barriers between microbes and enzymes and their substrates and controlling food web interactions. The current hypothesis of aggregate hierarchy concept (Tisdall and Oades, 1982) is based on free primary particles that are bound together into microaggregates (50-250 μm) by persistent binding agents (e.g. humified OM). These stable microaggregates are bound together by temporary (i.e. fungal hyphae and roots) and transient (i.e. microbial- and plant-derived polysaccharides) binding agents into macroaggregates (>250 μm), while in turn new microaggregates are predominantly formed within macroaggregates. Aggregate physical protection is further indicated by the positive influence of aggregation on the accumulation of SOM (Six et al., 2002). According to Skjemstad et al., (1996) the physical protection of chemically and recalcitrant organic matter within organo-mineral complexes and also charcoal formation are rather thought to be mainly the cause of long-term (decades to millennia) soil organic carbon sequestration mechanisms.

Chemical stabilization (2) of organic matter is the chemical or physico-chemical binding between organic matter and soil minerals (silt and clay particles in size) (Six et al., 2002). In soils with high clay content within the same climatic area and under identical annual organic matter input, a slower SOM turnover, larger microbial biomass and more organic matter accumulation are expected (Müller and Höper, 2004). This is because the mineral fraction has a profound effect on the quantity and quality of organic matter in soils due to the adsorption of organic matter on mineral surfaces. Different mineral species, such as silicate layers, primary or pedogenic oxides, are important for organic-mineral bonds (Schulten and Leinweber, 2000).

Biochemical stabilization (3) is the stabilization of soil organic matter due to its chemical composition (e.g. recalcitrant compounds such as lignin and polyphenols) and through chemical complexing processes (e.g. condensation reactions) in soil (Six et al., 2002). Humified organic matter represents the most persistent pool of soil organic matter with mean residence times of several hundreds of years (Piccolo, 1996). By humification, the plant residues are transformed via biophysical processes into more stable forms (humus). Thus, humification and degradation processes result on the one hand in the loss of structurally identifiable materials (Chefetz et al., 2002), and on the other in a gain in of stabilized organic compounds.

Since the soil is considered a limited and not expandable resource, the harmonizing ways of supporting soil function, which are often concurrent in the same area, becomes a crucial issue in terms of sustainability, in which political aspects are tending to prevail over scientific ones. As a consequence, all forms of soil management must be sustainable, namely they need to be conservation-minded instead of focused on environmental exploitation. Judicious management of croplands, forests, grasslands, and restored lands is the key to any potential C sequestration in the soil (Lal, 2002). Land-use controls the balance and transfer of C in terrestrial systems (Lal et al., 2003; Smith, 2004), the magnitude of soil disturbance and the amount of residue incorporated in the soil, factors that impact on organic carbon dynamics.

Several researchers have reported the effect of different plant species or fertilization effect on soil organic matter content, quality, and/or turnover. Plant litter is the main reserve for the formation of organic matter in the soil. The amount of organic residues from a plant, its composition and its properties are essential factors controlling the formation of soil organic matter and the processes of humification in terrestrial ecosystems (Coòteaux et al., 1995) Microbial biomass also comprises a significant fraction of the organic matter, and microbial residues in the soil are particularly important for the formation of humus (Haider, 1992).

Decomposition of organic residues depends on their composition, which may include lignin, phenolic compounds, aromatic compounds, sterols, and lipids concentrations. Furthermore, the lignin content and C/N ratio are the parameters that mainly affect the decomposition of SOM (Melillo et al., 1982 and Martens, 2000). For example, it is known that the lignin content is positively correlated with soil organic carbon concentration (Lamlom and Savidge, 2003), due to lignin resistance against microbial decomposition. Only a limited group of fungi (white-rot fungi) are able to completely decompose lignin to CO_2. Other fungi (soft rot and brown rot fungi) induce

structural changes in lignin, but they are not able to perform a complete mineralization (Kögel-Knabner, 2002). The organic residues with high lignin content are important to soil structure development; thus, lignin is associated with stabilizing and binding particles in aggregates (Magill and Aber, 1998) as well as with macroaggregation (Monreal et al., 1997). Amlung et al., 2002 found that on the surface of macroaggregates lignin is more easily decomposed than within the aggregates due to a increased external microbial activity. This explains the greater soil organic carbon sequestration within macroaggregates, so the long residence times of lignin-rich organic residue can enhance long-term soil organic carbon sequestration in aggregates. It is evident, therefore, that a series of complex biochemical and physical interactions depend on organic residue composition.

Crop residues can improve soil quality through their impact on reducing the risk of soil erosion (improving the physical protection of organic matter), stabilizing soil structure and providing energy for microbial processes (Indoria et al., 2017). The increase in organic matter content in the soil reduces erosion (by both wind and water), increases water availability and enables functional rebuilding of the microbial pool (microflora and microfauna). Min et al. (2003) reported that alluvium soil under cover crops, such as alfalfa, has higher soil aggregation induced by high crop root mass and easy litter decomposition, and thus enhances soil organic carbon sequestration. Jia et al (2006) suggested that the low C/N ratio in alfalfa residue might cause an acceleration of soil organic matter mineralization leading to C depletion, due to a more favourable C/N ratio for microbial biomass activity.

In agricultural land, the use of fertilization is common and the addition of nitrogen has been used to increase in tree growth, particularly for orchards such as walnut. Application of inorganic fertilizers results in higher soil organic matter accumulation and biological activity (Brar et al., 2015). Recently it has been shown that the addition of N in different forms (nitric, ammonium and urea) may have direct effects, mediated by plants, on the structure and activity of microbial communities (Giagnoni et al., 2016). Indirectly, fertilization can affect organic matter accumulation through root development and aggregate formation. Root developments is in fact a primary producer of SOM while, on the other hand, organic skeleton is able to mesh soil particles together, building aggregates. Ponder (1997) performed an in-depth review on walnut fertilization and reported that responses to added nitrogen can be quite variable. He concluded that it is not uncommon to see little or no response to fertilization, especially on good walnut sites. As already described above plants residue application is an important way to maintain soil productivity; thus, for example, walnut

leaves can be returned to the soil and their decomposition will improve soil fertility. Ma Hong-ye et al. (2016) analyzed the effect of foliar decomposition (Walnut Juglans sealed Dode) on the biological soil properties in order to determine whether the walnut leaf can be returned to soil or not and to obtain efficient decomposition conditions. They observed that adding walnut leaves to the soil decreases soil pH and increases nutrient contents, microbial quantity and enzyme activities.

1.3 Soil functionality through microhabitats - Aggregatusphere

Most ecological processes in agroecosystems and natural systems have their main dynamic control center in the soil (Lavelle, 2006), and more specifically in soil aggregates. The size, quantity and stability of soil aggregates reflect a balance between aggregate formation factors (organic fertilizer, soil microfauna and soil microflora, plant residue input, etc.) and other destructive depleting factors (i.e., deep tillage, soil erosion, etc.) (Six et al., 2002). Aggregates contribute to determining soil structure and from the physical space in which biotic and abiotic processes drives soil functionality. Soil structure is recognized as controlling many processes in soils. It regulates water retention and infiltration, gaseous exchanges, soil organic matter, nutrient dynamics, root penetration, and susceptibility to erosion. Soil structure also constitutes the habitat for a myriad soil organisms, thus driving diversity of these and regulating their activity (Elliott and Coleman, 1988). As important feedback, soil structure is actively shaped by these organisms, thus modifying the distribution of water and air in their habitats (Bottinelli et al., 2015; Feeney et al., 2006; Young et al., 2008). Kibblewhite et al. (2008) proposed the concept of soil health as a the direct expression of the dynamic combination of microbial groups of soil, which, in turn, depends on the physical and chemical conditions of the habitat within the soil.

The concept of "*aggregatusphere"* was been defined by Beare et al. (1995) and reviewed by Yakov Kuzyakov (2015) as aggregate-surface. According to Kuzyakov (2009 and 2010), aggregate-surface falls within the four "microbial hotspot groups" (rhizosphere, detritusphere, biopores and aggregate surface), where by hotspot he means a small soil volume with much faster process rates and much more intensive interaction between pools than under average soil conditions (Kuzyakoy 2009, 2010). The aggregatusphere is characterized by aggregated particles of different sizes and structural state related to porosity, forming a habitat for microorganisms and mesofauna. The primary boundaries of this sphere are those that limit the exchange of biota, solutes and gases across aggregate surfaces, investigate caracteristics that depend on the scale-size. The key concept here is that soil provides a living space for the biota (habitat), which is strongly related to the architecture

of the pore networks. Thus, the physical porosity framework defines the spatial and temporal dynamics of gases, liquids, solutes, particulates and organisms within the matrix, and without such dynamics there would be no function. The walls of soil pore networks provide surfaces for colonization, and the enormous range in pore sizes create the physical protection from predators, as does organic matter from microbial decomposition (Lee and Forest, 1991)

The soil architecture defines the microhabitat; therefore biotic and abiotic processes are influenced by aggregate size distribution, stability and pore space among and inside soil aggregates. A detailed understanding of microstructure can thus provide information on soil. Aggregation is conceptually viewed as three-stage hierarchical organization of the soil solid phase, each stage involving characteristic binding agents. Primary particles (< 20 μm) are bound together into microaggregates (20–250 μm), which are bound together to form macroaggregates (> 250 μm). Follow-up studies have favoured a different sequence of aggregate formation: macroaggregates can form around particulate organic matter, then microaggregates are released upon breakdown of macroaggregates (Angers et al., 1997; Oades, 1984). The bonds within microaggregates are supposed to be more persistent than those among macroaggregates (Tisdall and Oades, 1982). This hierarchical order, responsible for micro- and macro- aggregate formation, is identified in soils where soil organic matter is the major binding agent, but can be found neither in oxide-rich nor in sandy soils (Christensen., 2001; Oades and Waters., 1991; Six et al., 2004).

The “aggregate hierarchy” and “porosity exclusion” hypotheses typically postulate that soil organic carbon concentration and porosity will decline with decreasing aggregate size (Dexter, 1988; Tisdall and Oades., 1982), but soil organic carbon in micro-aggregates will be more stable and resistant to degradation. This stabilization of soil organic carbon in soil aggregates is believed to result principally from aggregate architecture and the protection of soil organic carbon results from microbial decomposition through formation of clay–organic carbon complexes (Sollins et al., 1996). Several investigations have found that the turnover of soil organic carbon is more rapid in macroaggregates than in microaggregates (e.g. Besnard et al., 1996; Six et al., 2002). Franzluebbers and Arshad (1997) concluded that microbial biomass and basal respiration were higher in macro- than microaggregates in Alfisols. Further, Fernandez et al. (2010) and Noellemeyer et al. (2008) reported that 1–4 mm aggregates had higher respiration than <1 mm aggregates in Mollisols. Thus, the association of soil particles and their spatial arrangement play a key role in organic C dynamics, but this role can vary according to aggregate size, as each aggregate possesses its own characteristic properties.

1.4 Biophysical parameters influencing soil functionality

Interaction of carbon with chemical, physical and biological soil properties make carbon content an important feature in soil quality assessment. The distribution of biological, biochemical, chemical and physical properties and the interaction thereof, in one word the distribution of biophysical properties, allows one to assess soil functions (since they often cannot be directly measured). They can be both qualitative and quantitative parameters. Recent trends in soil research attempt to integrate the biophysical properties mainly because the single properties do not precisely align with the various soil functions (Doran and Parkin, 1994).

In this work, we determine the different biophysical properties in order to characterize the soil habitat. The habitat includes the physical location, as well as the characteristics of the habitat that influence the growth, activities, interactions and survival of organisms. The habitat occurs on a microscale and therefore has been referred to as a microhabitat. The spatial characteristics of the microhabitats must be considered in describing the activity of soil microorganisms. Thus, it is important to highlight that the microbial component of soils is very sensitive than physical and chemical attributes to environmental changes and soil management.

According to (Schoenholtz et al., 2000), the hydrological processes like erosion, aeration, runoff, infiltration rate and water holding capacity are correlated with physical parameters. Soil texture is very stable over time and contributes to the balance between water and gases. Hence, for represent the effects of soil use and management on the water/air relationships the physical parameters (i.e. soil texture, aggregation, moisture, porosity, and bulk density) are important. The soil's physical attributes affecting water availability and aeration will also affect biological properties (such as soil microbial activity), since the content of available water is a determining factor of microbial activity in the soil (Geisseler et al., 2011). Thus, the loss of soil microbial activity due to water limitations can lead to loss of soil tasks like synthesis and mineralization of SOM and thereupon effect biogeochemical cycles. Organic matter, specifically soil carbon, transcends these property categories and is the most widely recognized parameter influencing soil quality, as it is associated with a large part of soil functions. Also physical and chemical parameters are commonly used as indicators for soil quality, which can give indication as to the soil capacity for upholding high yield crops (Gil et al., 2009). Chemical soil properties are related to the soil's capacity to retain chemical elements or compounds harmful to the environment and to provide nutrients for plants growth. Soil chemical parameters have been traditionally used for assessment of potentially available nutrients for crops, and are based on worldwide well-established analytical methodologies. Among them,

organic matter, pH, available nutrients, and some potential hazardous chemicals have been used to establish levels of soil health.

In forest or agricultural soils, the soil basal respiration is an important biological parameter, thanks to its close relationship with soil organic matter. A decrease in organic carbon inputs into the soil has been shown to reduce soil respiration and impacting management affects soil biological activity by depressing it. The metabolic quotient (qCO_2) is an index often used to measure biological activity, given by the amount of CO_2-C released per unit of microbial biomass in time. It represents the metabolic status of soil microorganisms (Anderson and Domsch, 1993). Low qCO_2 values usually indicate both a favorable microbial habitat and input of hardly degradable organic carbon that slows down microbial activity (Anderson and Domsch, 1989).

Soil microbial properties are broadly used thanks to their high sensitivity and because they give integrated information concerning environmental factors (Gómez-Sagasti et al., 2012). In defining suitable biological indicators, able to give information about soil species diversity, a number of methods may be used in order to measure both abundance and diversity/function. As sensitive indicators of soil quality have also been also suggested soil enzyme activities (Gianfreda and Bollag, 1996; Calderon et al., 2000; Drijber et al., 2000; Nannipieri et al., 2002). Nonetheless, it is possible to use them as an indirect measure of functional diversity, since soil enzyme activities link the microbial population with nutrient dynamics (Sinsabaugh and Moorehead, 1994), and since they differ between soils, (Caldwell, 2005).

Biochemical and biological properties are thus recognized to be highly sensitive towards changes in soil. Physical and chemical features change less over time, and can describe soil evolution as a consequence of changing conditions or activities as a medium or long-term response. However, they define the physical and chemical environment in which microbiota acts. Individual usage of these properties cannot convey full understanding of the ecological processes occurring in the soil, thus, the integration among indicators seems to be a more appropriate approach to assessing soil status.

1.5 Research aim

The aggregates that contribute to determining the structure of the soil form the physical space and habitat in which soil microorganisms live and perform their tasks, regulating soil system functioning. Consequently, studying the biophysical properties of soil aggregates can be an effective tool for assessing the influence that different types of soil management may have on soil functionality. The C sequestration function is one of the main challenges for soil management. We therefore carried out an interdisciplinary study on the biophysical properties of aggregates with **the aim of assessing the effect of management on the biophysical properties of different aggregate size classes, relating them to soil C accumulation**.

In this study, we investigated the biophysical parameters of soil macroaggregates (4000-1000 μm), mesoaggregates (1000-250 μm) and microaggregates (<250 μm) in mountain and plain agroecosystems. The mountain agroecosystem was composed of a soil under alfalfa crop and a soil under an oak wood. The plain agroecosystem consisted of the soils within an experimental walnut orchard characterized by differing urea distribution: one site was fertirrigated (90 kg urea ha^{-1}), one site received granular urea (90 kg urea ha^{-1}), and one site was the control (without urea addition).

In chapters 5 and 6 we study the effect of management on macro-, meso- and microaggregates through the chemical, biological and physical parameters of each aggregate size class in mountain and plain agroecosystems, respectively.

1. Hypothesis – We assumed that different aggregate fractions represent different microhabitats, each of which has specific biophysical properties, regulating the carbon sequestration function.

In Chapter 7, we study the physical and chemical changes in macro- and mesoaggregates through optical microscopy and SEM-EDS analysis on thin sections.

2. Hypothesis – We assumed that the physical location of organic matter and the spatial relationship between organic matter and pores should be different in the two aggregate classes and consequently the features of organic matter (e.g., morphological form and chemical composition) should differ.

2. MATERIALS

2.1 The study areas

The study covers two different agroecosystems: a mountain and a plain agroecosystems in the Emilia Romagna region (northern Italy). In the mountain agroecosystem we selected two soils in Monzuno in the Appennine mountain close to Bologna (Fig 1). One soil was under oak wood (MO-W site) and the other one under alfalfa (MO-A site). More details on land-use are given in paragraph 2.2. Monzuno is located between the slopes of the Savena, Setta and Sambro river valleys and emerges in the central part where the Monte Venere reaches an altitude of 621 m a.s.l. In the plain agroecosystem we selected three soils situated in an experimental walnut orchard located in Bordone (Cadriano) in the province of Bologna (Fig 1). Cadriano is a town belonging to the municipality of Granarolo dell' Emilia in the metropolitan city of Bologna in the Emilia-Romagna region. The Cadriano is located at altitude 32 m a.s.l. The three soils in the experimental walnut orchard were differently N fertilized: one was the control (PL-CONTR site), with 0 input of N, the other two sites received 90 kg ha^{-1} of N as urea distributed by fertirrigation (PL-FERT site) or in granular form (PL-GRAN). More details are given in paragraph 2.2.

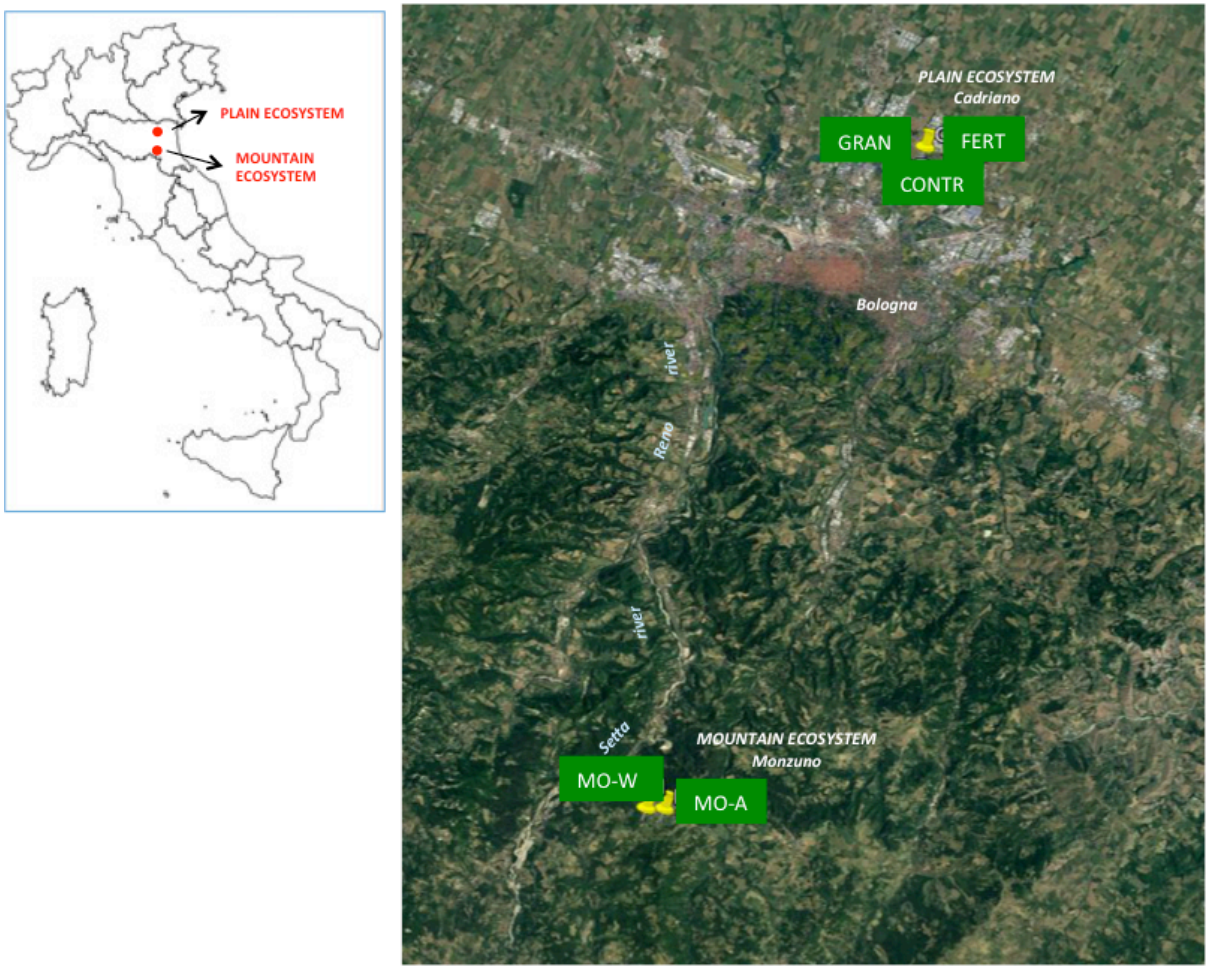


Figure 1. Geographical location of sampling sites in Emilia Romagna. MO-W and MO-A represent the oak wood and alfalfa sites, respectively. PL-CONTR, PL-FERT and PL-GRAN are the control, fertirrigated and granular walnut sites, respectively.

The climate of the mountain and plain agroecosystem is temperate and subtropical wet climate, respectively, according to the Kopper-Geiger classification system. In 2014, the total annual rainfalls and mean air temperature registered by the Arpa meteorological station were 944 mm and 10.3 °C in the mountain agroecosystem and 737 mm and 14°C in the plain agroecosystem respectively. Their monthly trends are reported in Fig 2.

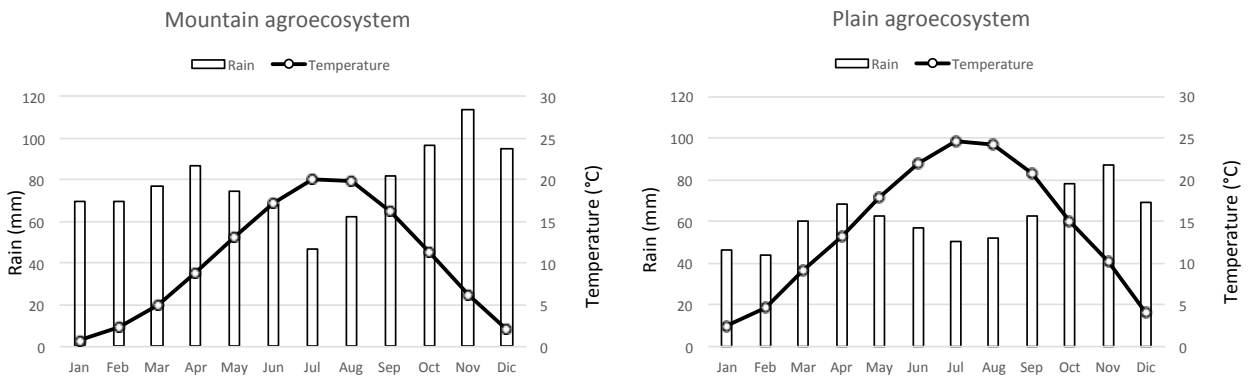


Figure 2. Monthly trends of temperature and rainfalls registered at the two agroecosystems over 2014.

The soils of oak wood and alfalfa sites in the mountain agroecosystem were classified as Typic Eutrudept and Aquic or Vertic Eutrudept, respectively (Soil Survey Staff, 2014), according to the regional soil map of the Servizio Geologico, Sismico e dei Suoli della Regione Emilia Romagna (2015). These soils formed on limestone-marl and pelitic-sandstone stratifications. A common feature of the soils of these environments is the rather complete decarbonation of the profile. The main differences between the soils derive from the morphology of the soil surface and the vegetative cover that lies on it (Vittori Antisari, 2005). Both soils show a moderate degree of differentiation of the profile also due to erosive phenomena for water runs off and landslide events (Vittori Antisari, 2005).

The soils of the plain agroecosystem were classified as Udifluventic Haplustepts (Soil Survey Staff, 2014), according to the regional soil map of the Servizio Geologico, Sismico e dei Suoli della Regione Emilia Romagna (2015). These soils were characterized by the presence of conoids, i.e. sedimentary bodies consisting of a clastic sediment accumulation, that fall into the category of alluvial sediments and result from the sedimentation of material transported by a stream when the river current slows and expands. The soils of this agroecosystem have clay-loam or silty-loam texture and are characterized by a sequence of slightly developed horizons, in which signs of alteration of primary minerals are observed (Servizio Analisi e Consulenza Terreni 2010).

2.2 Agronomic management of agroecosystems

The soils in the mountain agroecosystem are used for both agricultural and forestry purposes. In the agricultural soil, specialized grassy crops are widespread, while forests are mainly used for wood production. The studied sites are representative of the two typical soil usage in this agroecosystem.

We sampled two sites: the first site was under oak wood (MO-W) exploited for firewood with 16 years cutting-cycle (Italy, 44° 16'29''N; 11°14'53''E), and the second site was a 5-year-old not fertilized alfalfa crop (MO-A) (Italy, 44° 16'28''N; 11°15'25''E) (Table 1). At the time of soil sampling, both sites had reach the end of their silvo/cultivation-cycle.

Agroecosystems	**Site**	**Site description**	**ID**
Mountain	Oak wood	cut 16 years ago	MO-W
	Alfalfa	5-year-old crop	MO-A
Plain	Walnut Control	without urea addition	PL-CONT
	Walnut Fertirrigated	fertirrigated	PL-FERT
	Walnut Granular	granular by localized surface	PL-GRAN

Table 1. List of the investigates sites

In the region, the plain agroecosystem mainly consists of crops and orchards (mainly pomacee and vineyards). In this study we avoided agricultural sites subject to annual tillage operations strongly affecting soil aggregation (Bronick and Lal, 2005) and we selected a walnut (*Juglans regia* L.) grove of the cv. Lara located in the experimental farm of the University of Bologna. The experimental walnut was in place since 2001 and it consists of a randomized block scheme of 5 rows of 20 plants each, with 5 replicates (Fig 3). Four thesis occurred in the experimental walnut (control, addition of compost, addition of urea by fertirrigation and localized addition of granular urea; Fig 3). Three out of four thesis have been investigated in this study (Table 1). The three investigated thesis differ for different urea distribution:

- Control (PL- CONT) (without urea distribution)
- Fertirrigated (PL-FERT) receives urea by underground irrigation (90 kg urea ha^{-1})
- Granular (PL-GRAN) receives urea by localized fertilisation (90 kg urea ha^{-1})

The experimental walnut is in a randomized block design, with five replicates, arranged in five adjacent tree rows. Experimental unit is consisting of four consecutive trees, were randomly

distributed within each row. Along the perimeter of the experimental walnut, the plants of the external rows have been left out in order to avoid any edge effects (Fig 3). The distance between the two outer rows and the side ditches was 3.5 m. In the installation year, plowing was carried out, followed by the drawing up of the irrigation system and the planting of the plants.

As regards to fertilization, an equivalent dose of commercial urea containing 46% of N was distributed annually to each plant. Thus, since 2001, 1200 g of urea has been distributed for fertirrigated and granular treatment subdividing in two doses for year (600 g in April/May and 600g in October).

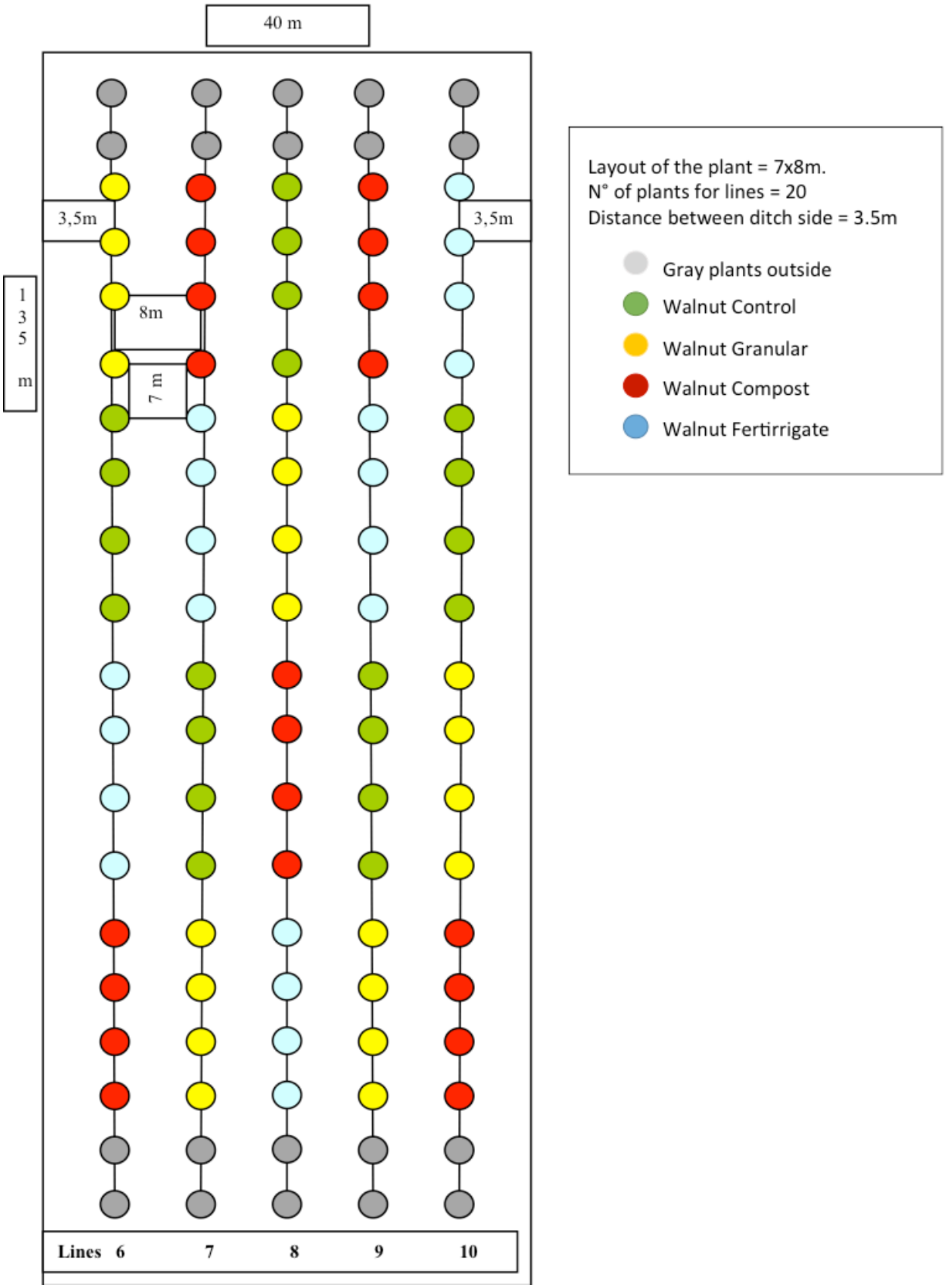


Figure 3. Scheme of experimental walnut

3. METHODS

3.1 Soil sampling

In the mountain agroecosystem (oak wood MO-W and alfalfa MO-A), two pits were dug in a representative area of each site and the 0-20 cm topsoil, corresponding to A horizon for oak wood sites and Ap horizons for alfalfa site, was collected from each pit. For the plain agroecosystem the same sampling scheme was repeated and 0-20 cm topsoil (corresponding to Ap horizons) was collected from the two pits opened in each site and, to avoid the possible mulching effects, the sampling was done along the plant rows. All soil samples were air dried at room temperature and sieved with a series of sieves in order to separated three different aggregate fractions: macroaggregates (4750-850 μm), mesoaggregates (850-250 μm) and microaggregates (<250 μm). The weight of each aggregate class was recorded. An aliquot of each aggregates fraction were further milled to <0.5 mm size.

3.2 Chemical parameters

On the three fractions of aggregates the pH was determined potentiometrically in a 1:2.5 (w:v) soil-deionised water suspension (Van Reeuwijk 2002). Total organic carbon (C_{org}) and total nitrogen (N) content were determined on air dried, finely ground soil aggregates subsamples (ground to <0.5 mm) by an elemental analyser (CHNS-O Elemental Analyser 1110, Thermo Scientific GmbH, Dreieich, DE). The relative abundance of C and N stable isotopes was determined by continuous flow- isotope ratio mass spectrometry (CF-IRMS) using an isotopic mass spectrometer Delta V advantage (Thermo- Finnigam, DE). The values were then expressed as $\delta^{13}C$ and $\delta^{15}N$, as deviation in parts per thousand compared to the universal reference standard. The carbonate ($CaCO_3$) content was measured by the gas-volumetric determination of CO_2 released by the ground sample <0.5 mm with hydrochloric acid (Loeppert and Suarez, 1996). For this determination the Dietrich-Fruehling calcimeter was used. The total Al and Fe concentration (Al_t and Fe_t) was measured by ICP-OES (Spectro Ciros ^{CCD}, Germany) after HNO_3: HCl (1:3) microwave digestion of the sample.

3.3 Biological parameters

3.3.1 Microbial biomass carbon

On each aggregate class, microbial biomass carbon (C_{mic}) was determined using the chloroform-fumigation extraction method (Brookes et al., 1985). The equivalent of 10 g of oven dried aggregates was weighted and water content was adjusted to 70% of water holding capacity. The samples were then fumigated with ethanol-free chloroform for 24 h at room temperature in a desiccator. Fumigated and non-fumigated samples were dispersed in 40 mL of 0.5 M K_2SO_4 and extracted on an horizontal shaker at 250 rev min^{-1} for 1 h. Extracts were filtered through Whatman no. 42 filter paper and analyzed for the organic C content with an elemental analyser (TOC-VCPH/CPN, Shimadzu, Kyoto, JP). C_{mic} was calculated as organic C in the fumigated minus organic C in the non-fumigated soil extracts (C_{extr}). Similarly, N_{mic} was calculated as total nitrogen in the fumigated minus total nitrogen in the non-fumigated soil extract (N_{extr}).

3.3.2 Basal respiration and metabolic quotient

Microbial respiration was estimated according to Isermeyer (1952) for each aggregate classes. The equivalent to 10 g of oven dried aggregates, was weighted into airtight glass jars. Water content was adjusted to 70% of water holding capacity and samples were incubated at 25 °C for 3 weeks. Evolved CO_2 was trapped in plastic vials containing 2 mL of 0.5 M KOH and measured at 1-2-3-4-5-10-15-21days during the incubation. Trapped CO_2 was quantified by titration, after precipitation of carbonate with 4 mL of 0.75 M $BaCl_2$, using 0.1 M HNO_3. The CO_2 evolution of the 21st day was used as a measurement for the soil basal respiration (R_{basal}) and as cumulative respiration (R_{cum}) by varying the date of equivalent carbon weight in CO_2 (i.e. equal to 6 if the results were expressed in terms of C and equal to 22 if the results were expressed in terms of CO_2). Among the eco-physiological parameters metabolic (qCO_2) and mineralization quotients (qM) were calculated. The qCO_2 was determined as R_{basal}/C_{mic} (Dilly and Munch, 1998), the qM was calculated as R_{cum}/C_{org} (Pinzari et al., 1999).

3.3.3 Enzymatic assays

The activity of eight extracellular hydrolytic enzymes was studied (Tab 2). All the assays were conducted on all aggregates fraction samples of both agroecosystems.

Enzyme	Enzyme function	Substrate
β-1,4-glucosidase (β-GLU)	Cellulose oligomers into β-D-glucose	4-MUF β-D-glucoside
α-1,4-glucosidase (α-GLU)	Starch into α-D-glucose	4-MUF-α-D-glucoside
N-acetyl-β-glucosaminidase (N-AG)	Chitooligosaccharides into chitin oligomers	4-MUF-N-acetyl-β-D-glucosamide
β-1,4-xylosidase (β-XYL)	Xylooligomers into xylan	4-MUF- β-D-xyloside
β-1,4-cellobiosidase (β-CEL)	Cellulose into cellobiose	4-MUF-β-D-cellobioside
Arylsulfatase (SULF)	Organic S into sulfates	4- MUF-sulfate
Phosphomonoesterase (PME)	Phospate monoesters into phosphate	4-MUF-phosphate
Phosphodiesterase (PDE)	Phosphate diesters into phosphate monoesters	bis-4-MUF-phosphate

Table 2. Enzymes included in the study, abbreviations, catalyzed hydrolysis, and corresponding MUF model substrates* (MUF= 4-merthylumbelliferone).

Previously, kinetic experiments were carried out where substrate saturating conditions were determined for each sample and enzyme activity using different levels of substrate dilution, corresponding to 5, 20, 50, 100, 150, 200, 400, 600, 800, 1000 μM. The kinetic experiments were carried out with the aim to obtain the exact concentration of substrate corresponding to the maximum velocity of the enzymatic reaction. In table 3, we showed the concentration values used for each site.

Activity	Site				
	MO-W	MO-A	PL-CONTR	PL-GRAN	PL-FERT
β-GLU	400	300	500	500	400
α-GLU	400	400	500	500	500
N-AG	600	600	800	800	800
β-XYL	800	800	800	800	1000
β-CEL	400	200	600	800	800
SULF	2000	2000	2000	2000	2000
PME	800	800	1000	800	800
PDE	800	1000	800	1000	1000

Table 3. Substrate suturing concentration (μM) as determined by kinetic experiments

The saturating substrate concentrations established in the kinetic experiment were then used as substrate concentrations for the enzymatic activity assays. The activities were assayed using MUF (7-hydroxyl-4-methylcoumarin) conjugates following the study reported by Giacometti et al. (2013). A 0.5 M sodium acetate buffer solution was made by mixing sodium acetate trihydrate (analytical grade, crystalline, Carlo Erba) with deionized water. The pH was adjusted to 5.5 using glacial acetic acid (99.9 % v:v, Carlo Erba) (ISO/TS 22939, 2010). This buffer solution was then used to dilute standard, substrates and soil samples. To minimize variability due to reagents storage, substrates and standard solutions were prepared on the day of the assay. Freshly made solutions were kept away from light until use. To avoid microbial contamination, glassware, buffers and deionised water were sterilized in autoclave (121 ± 3 °C for 20 min) before usage (ISO/TS 22929, 2010). Each substrate was pre-dissolved in dimethyl sulfoxide (DMSO, SIGMA). Sodium acetate buffer was then added to give the desired final concentration. Five mM 7-hydroxyl-4-methylcoumarin (MUF) standard solution was prepared in methanol and water (1:1, v:v). This stock solution was diluted to 1.00, 2.00, 4.00, 10.0, 20.0, 30.0, 40.0 μM in sodium acetate buffer.

Soil samples corresponding to 2 g of oven dried soil were weighted into sterilized Pyrex tall-form 150 mL becker. One hundred mL of 0.5 M acetate buffer were added and mixed using an Ultra Turrax IKA for 2 min at 9000 rpm (IKA-Werke, Staufen, DE). A magnetic stir bar was then added and soil was kept under continuous stirring. The entire procedure of soil samples processing was staggered so that the time between soil slurry preparation and subsequent substrate addition never exceeded 40 min. Flat-well black polystyrene 96-well micro-plates with a well capacity of 350 µL were used throughout the experiment (Greiner Bio-One, Frickenhausen, DE). Buffer, soil slurry, standard solutions and substrate solutions were dispensed in the micro-plates in the following the same order. First 100 µL of sodium acetate buffer were dispensed in the wells that served as soil controls and substrate controls. Next 50 µL of sodium acetate were dispensed in the wells that served as quench controls. Then, 150 µL of sodium acetate buffer was added in the wells that served as reference standards. Soil slurry aliquots of 100 µL were then withdrawn from the soil suspension under continuous stirring and dispensed in the wells that served as quench controls, soil controls and soil assays. After all the soil slurries included in the assay were processed and dispensed, 50 µL of MUF standard solutions were dispensed into wells that served as quench controls and reference standards. Lastly, 100 µL of substrate solutions were dispersed into wells that served as substrate controls and soil assays. The total volume of the reaction mixture was 200 µL. Eight analytical replicates were used for soil assay and substrate controls wells. Four analytical replicates were used for reference standards, quench controls and soil controls wells. The addition of the substrates was considered the start of the incubation period. The micro-plates were covered and incubated in the dark at 30 °C. The fluorescence intensity was measured using a microplate fluorometer (infinite200, TECAN, Männedorf, CH) with 365-nm excitation and 450-nm emission filters. Measurements were taken immediately after the plate set-up and from then on every 30 min over a 3 h incubation period. Before each reading the microplates were shaken for 5 s in order to homogenize the reaction mixture. Enzyme activities were expressed in nmol product h^{-1} g^{-1}. Rates of fluorescence increase rather than absolute amount of fluorescence at the end of the incubation period were used for the calculation. Rates of fluorescence increase were converted into enzyme activity according to the following equations (adapted from Marx et al., 2001 and German et al., 2011):

$$(1) Activity\ (\text{nmol MUF g}^{-1}\text{h}^{-1}) = \frac{\text{Net fluorescence (RUF min}^{-1}\text{)x 100 (mL) x 200 (µL)x 60 (min h}^{-1}\text{)}}{\text{Emission coefficient (RUF/µmol L}^{-1}\text{) x 100 (µL) x Soil dry mass (g)}}$$

Where:

$$(2) Net\ Fluorescence = \left(\frac{\text{Assay slope } - \text{ Soil control slope}}{\text{Quench coefficient}}\right) - \text{Substrate control slope}$$

Emission coefficient (RUF/ µmol MUF L^{-1}) = Reference standard curve slope

$$(3) Quench\ coefficient = \frac{\text{Quench controls curve slope (RUF/µmol L}^{-1}\text{)}}{\text{Reference standards curve slope (RUF/µmol L}^{-1}\text{)}}$$

RUF = Relative unit is of fluorescence

The specific activities of the enzymes are calculated dividing enzyme activities by C_{mic} (Waldrop et al., 2000) or by C_{org} (Trasar-Cepeda et al., 2008).

3.3.4 Microbial molecular diversity

Genomic DNA was extracted from 250-300 mg of dried soil using the PowerSoil DNA kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was eluted with 70 µL of 10 mM Tris-HCl pH 8.0. The purity and quantification of extracted DNA was determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 196 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). Extracted DNA (DNA_{tot}) was stored at -20 °C. The DNA_{tot} were calculated by dividing DNA_{tot} by C_{mic} (DNA_{tot}/ C_{mic}).

PCR amplification was performed by using the extracted DNA as a template with the universal fungi primer pair Euk1A-fw (5'-CTGGTTGATCCTGCCAG-3') and Euk 516-rev (5'-ACCAGACTTGCCCTCC-3') with a 40-bp GC clamp attached to it 5' (5'-CGCCCGGGGCGCGCCCCGGGCGG GGCGGGGGCACGGGGGG-3') (Zhao and Xu 2012).
The 30 µL PCR reaction contained 15 µL of 2X HotStarTaq Master Mix Kit (HotStarTaq Plus DNA Polymerase 5U μL^{-1}, PCR Buffer (with 3 mM $MgCl_2$), and 400 µM each dNTP) (Qiagen;

cod: 203445), 0.30 µL of each primer Euk1A-fw and Euk516-rev (20 µM) 0.1 µL bovine serum albumin (20 mg mL^{-1}) (Fermentas), 3 µL DNA template and sterile MilliQ water to reach the 30 µL final reaction volume. Soil DNA was amplified using the Verity Thermal Cycler (ThermoFisher) with the following program: 95°C for 15 min; 40 cycles consisting of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 130 s; followed by a final extension cycle of 72 "C for 10 min.

The size of the PCR products (~600 bp) were checked by analysing 5 µL of amplified products by 1.5% agarose gel (w v^{-1}) electrophoresis and ethidium bromide staining.
The fungal community analysis was carried out by DGGE, according to Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad, Richmond, CA, USA), employing 7% polyacrylamide gels with a denaturing range of 30-40%. The electrophoresis was run at 55 V for 16 hours at 60°C. Gels were stained in a solution of 1X SYBR-Green (Sigma–Aldrich) in 1X TAE for 20 min and their images captured in UV transillumination with Gel Doc™ XR apparatus (Bio-Rad, Richmond, CA, USA). Patterns were normalized by including a ladder with PCR products obtained from samples of the superficial horizon of each site.

Comparison and cluster of DGGE profiles were carried out using the unweigthed pair-group method with the arithmetic average (UPGMA) clustering algorithm based on the Dice coefficient with an optimization of 1% and resulted in a distance matrix (Gel Compare software, version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). Microbial diversity was analyzed with Gel Compare 6.6 for the following parameters: Shannon-Wiener index (H') and band evenness (J'), calculated according to Hill et al. (2003).

3.4 Physical parameters

3.4.1. Texture analysis

The clay (<2 µm) and silt (2-50 µm) particle distribution was obtained by the pipette method after dispersion of the sample with a sodium hexametaphosphate solution (Gee and Bauder 1986). The coarse sand particles (2-0.2 mm in size) were determined by wet sieving. All the fractions were expressed as g kg^{-1} and the fine sand particle (0.2-0.05 mm) were obtained by subtraction to 1000 the sum of clay+silt+corse sand content.

3.4.2 Porosity and pore size distribution by mercury intrusion porosimetry (MIP)

The pores volume was determined using a Hg porosimeter (Porosimeter 2000 WS equipped with a Macropore unit 120, CE Instruments, Rodano, Italy) by step measuring of the pressure required to force Hg into the pores and of the volume of intruded Hg at each step. Mercury intrusion was performed up to 200 MPa of applied pressure. Assuming that the pores are cylindrical, the relation between equivalent pore radius (R expressed in μm) and applied pressure (P expressed in MPa) is described by the equation (Washburn, 1921):

$$(4) 2R = \frac{-4S\cos Q}{P}$$

Where S is the surface tension of mercury and Q its contact angle with the soil material. The value of S and Q were taken as 0.480 N m^{-1} and 141.3°, respectively. For irregularly shaped pores, the ratio between the pore cross-section (related to the pressure exerted) and the pore circumference (related to the surface tension) is not proportional to the radius and depends on the pore shape. The equivalent pore size calculated by the equation (4) will thus be lower than the exact pore radius. However, although soil pores are rarely cylindrical in shape, the Washburn's equation is normally used to calculate the equivalent pore size from mercury porosimetry data (Lowell and Shields 1991). At the highest level of applied pressure, the smallest measurable radius was 0.0037 μm. The total volume of intruded Hg (i.e., total pore volume) was expressed on a mass basis (mm^3 g^{-1} V_{Hgtot}). From the data obtained by Hg intrusion, it was possible to calculate the specific surface area in m^2 g^{-1} (SSA) as ratio between the volume and the pore radius, applying sample cylindrical geometry model. Pore size distribution was also determined, considering five radius pore classes according to Greenland (1977): pores <0.005 μm and 1-0.005 μm were classified as residual pores, pores between 1-25 μm and 25-50 μm as storage pores and pores between 50 and 75 μm as transmission pores.

3.4.3 Aggregate thin sections: micromorphology observation, image analysis of aggregate pores and organic components

Aggregate thin sections (2.8 x 4.8mm) were obtained in the Piombino Laboratory, from the three different aggregate fractions of each site. Since the microfeatures in these thin-sections have been analyzed for their elemental composition by microanalysis performed by scanning electron microscope (SEM) equipped with EDS probe, these slides were not cover-slipped.

In order to study the thin sections, it was necessary to identify the area of interest (i.e., the most representative area of features in the thin sections). In this study, the area of interest represents the area of the single aggregate (within aggregate). This preliminary selection of area of interest highlighted the impossibility to study by optical microscopy the microaggregates (<250 μm aggregate size). This was because of small size of microaggregates that did not allow a clear outlined of the surface of each single aggregate distorting the data. So macro- and mesoaggregate were analysed. For macroaggregate thin sections, between 9 and 16 aggregates were analysed for each site, while for mesoaggregates between 23 and 41 aggregates were measured. In both cases, the edges of the thin sections and the areas of interest (within aggregate) with inside or near artificial bubbles have been avoided.

A general description of thin section was made at both 10x (whole thin-section) and more in detail at 20x (within aggregate) using a polarised microscope (Olympus BX51) under plane (PPL) and crossed polarized light (XPL). These conventional descriptions were made following the guidelines recommended by Stoops (2003) and were reported in supplementary material (Chapter 10, Table 26). At 20x magnification, the estimates of abundance of some fabric units (porosity and organic matter pedofeatures inside of aggregates) were made using abundance diagrams (Fitzpatrick, 1980). These estimates give a general information on samples and allow a direct comparison with quantitative results deriving from image analysis.

High-resolution images were captured at 40x using a digital camera, and connected to a computer equipped with an images framegrabber. Captured images were then available for computerised analysis carried out by AnalySIS v 510 (Olympus Soft Imaging Solutions GmbH) image analysis software. Image analysis provides quantitative information from scanned image. The image analysis was applied to calculate and characterize porosity and soil organic matter parameters in intra-aggregates. To measure porosity, multiple images of the same representative aggregates were taken under both PPL and XPL light. This was necessary to distinguish between pores and quartz, since both are translucent under PPL. These images were additively combined and the result inverted. The inverted images were multiplicatively overlapped with a natural light image to produce a composite binary image in which minerals were readily distinguished from voids, with minerals and soil matrix represented by black pixels and pores by white pixels. Total porosity ($porosity_{tot}$) and pore size distribution was measured according to different size classes (Cameron and Buchan, 2006): micropores (<25 μm), mesopores (25-50 and 50-75 μm) and macropores (75-100, 100-200 and 200-350 μm). The identification of organic residues was performed under PPL light, and

organic features were categorized as being either organ or amorphous in form (Babel, 1975). Once classified according to form, organic components have been further described according to the extent of their decomposition following the classification proposed by Fitzpatrick (1993). Organ fragments can be either fresh/living, moderately or strongly decomposed, while amorphous forms are strongly decomposed and are further described by their colour, with yellow-black indicating greater decomposition due to oxidative and microbial processes (Bullock et al., 1985). By applying these guidelines it was possible to achieve a systematic method for classifying the different organic components. Fig 4 shows a scheme of organic components classification used in this work.

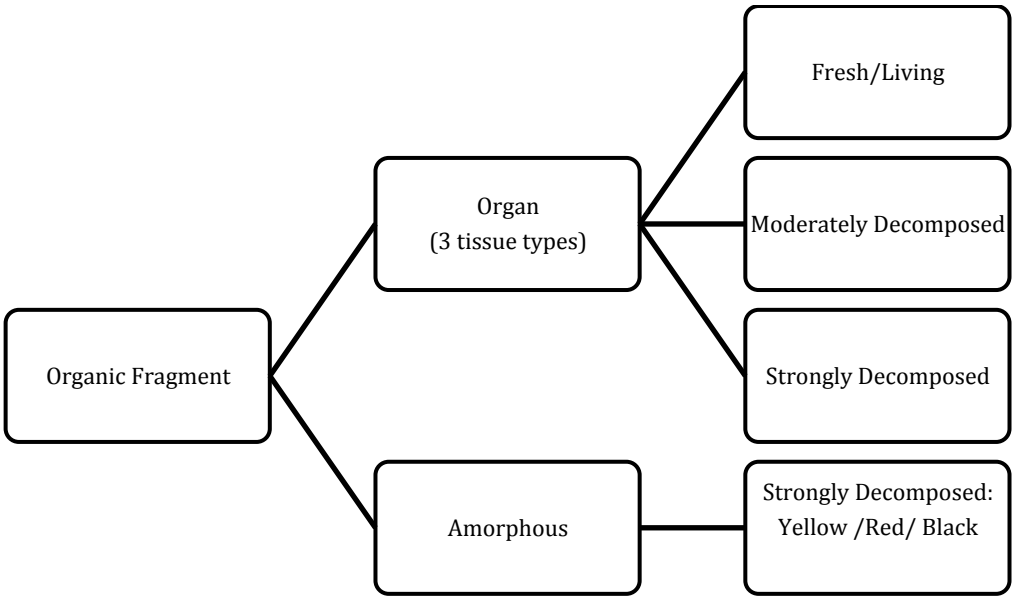


Figure 4. The process of organic matter classification.

Once each organic component in the representative area has been distinguished, a manual delimitation of each has been provided using image analysis software within PPL images. Images were thus segmented selecting for organic fragments, and measurements were made including the frequency and area of each class of organic features.

By exporting images obtained by organic components analysis into an image manipulation program (GIMP 2.6), organic features were colour coded according to form (Babel 1975) and decomposition (Fizpatrick 1993) and stacked upon the binary pore image thereby forming a map showing the distribution of organic matter in relation to soil pores. For each area of interest, it was thus possible to measure the perimeter (mm) of organic matter in contact to the pores (SOM-PORE map, in supplementary data Chapter 10, Table 27), which was then normalized with respect to the total surface of organic forms (mm^2). The obtained index, called exposure index (EI [mm^{-1}] calculated as

OM total perimeter in contact to pores [mm]/OM total surface [mm^2]), measures the degree of organic matter interaction with the pore system, and thus the potential organic matter occlusion in the aggregate matrix.

3.4.4 Aggregate thin sections: SEM-EDS analysis

Polished thin sections left non-cover slipped were analysed using an environmental scanning electron microscope (ESEM) and elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems (EVO MA15) linked to an Oxford Instruments INCA X-max detector with an 80-mm^2 SDD. For this work the principal element of interest was carbon, low vacuum conditions (>30 kPa) were therefore used to control charging without C-coating the sample. Optimal C detection was ensured using an accelerating voltage of 5-20 keV, a process time of 5.0, a working distance of 8.5 mm, a spot-size between 500-560. EDS analysis was performed at high magnifications (500-1,000x). The microanalysis was carried out for organic features at least on 100 point for each thin section. Data were normalized to 100%, giving a semiquantitive measure of elemental concentration. Thus elemental molar ratios are discussed in this thesis rather than absolute concentrations. We reported the values of the O:C ratio taking into account that the data obtained from this punctual analysis can be affected by the elemental composition of mineral phase (this includes silicates, silicate on oxides and oxides) interacting with the organic substances. As a result we also reported several other elemental ratios, as Al:C, Fe:C and Ca:C molar ratio.

3.5 Data treatment and statistical analysis

The chemical, biological and physical results were obtained by the arithmetic means of the values obtained by the two soils sampling (two field replications for each sites). The experimental data reported in chapter 5 and 6 are further transformed and expressed as weighted average values and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction of interest. In supplementary material (Chapter 10, Tables 22 to 25), the original data of the chemical, biological and physical characteristics of the aggregates fraction for each site were reported.

Differences among treatments were evaluated by the analysis of variance (ANOVA) and HSD Tukey's test. Before analysis, the homogeneity of variances was checked using the Levene test and the normality of data through the Shapiro-Wilk test. The DNA values were the result of the mean of analytical replications (three for each field replications), therefore we showed two values for each sites. These two values represented the field replications (1° sampling and 2° sampling). Similarly to chemical, biological and physical results, DNA values reported in chapter 5 and 6 are expressed

as weighted mean. Variations among samples were evaluated by the analysis of variance (ANOVA) and HSD Tukey's test. For the micromorphology observation of thin sections (chapter 7), a two-way ANOVA analysis of variance was carried out on porosity, organic features and EI data considering both site and aggregate fraction. The correlation between micromorphological properties and chemical and biochemical properties of aggregates was evaluated using the Spearmann coefficient. For SEM-EDS analysis, variations among samples were evaluated by the analysis of variance (ANOVA) and HSD Tukey's test. The threshold used for significance in all statistical tests was set at 0.05. All data treatments were carried out using SPSS software package (SPSS Inc. Chicago, IL).

4. RESULTS AND DISCUSSION

In the following chapters 5 and 6, we have investigated how different management can affect the biophysical properties of aggregate fractions related to the carbon sequestration function, in mountain and plain agroecosystems (chapters 5 and 6, respectively). The assumption was that different aggregate fractions represent different microhabitats, each of which has specific biophysical properties related to soil functionality (e.g soil carbon sequestration). To assess the effect of management on the biophysical properties of each aggregate fraction, we present the data obtained from the chemical, biological and physical parameters of macro-, meso- and microaggregates from investigated mountain and plain agroecosystems.

Taking into account the different response to soil management and the aggregate formation process between macro- and mesoaggregates (which we will observe in the chapters 5 and 6), in chapter 7 we presented a study focusing on macro- and mesoaggregate classes. We hypothesized that the physical location of the organic matter and the spatial relationship between soil organic matter and pores differed between the two aggregate classes and so, consequently, did the features of organic matter (e.g, morphological form and chemical composition). This information should be useful when it comes to understanding the effect of soil management and aggregation on soil functionality related to C dynamics.

5. MANAGEMENT EFFECTS ON BIOPHYSICAL PROPERTIES OF DIFFERENT AGGREGATE FRACTIONS (MICROHABITATS) IN A MOUNTAIN AGROECOSYSTEM

5.1 Aggregate size distribution and chemical parameters

In the oak wood site the macro-, meso- and microaggregates accounted for the 91, 6 and 3% of total soil mass, respectively (Table 4). In the alfalfa site they accounted for the 80, 11 and 9%. The macroaggregates represented thus, in both sites, the largest part of the soil mass.

In both sites, the largest part of soil organic C was in the macroaggregates (Table 4), which contained 40.9 and 8.8 g kg^{-1} in the oak wood and alfalfa sites, respectively. In the meso- and microaggregates the content of organic C was lower and ranged from 1.2 to 2.9 g kg^{-1}. A similar distribution pattern was observed for total N, whose amount in the macroaggregates was 3.54 and 0.85 g kg^{-1} in oak wood and alfalfa sites, while it ranged from 0.24 to 0.10 g kg^{-1} in the meso- and microaggregates. The organic C to total nitrogen ratio (C/N) varied from 11.6 to 12.4 in the oak wood site, and from 10.5 to 10.9 in the alfalfa site, and it was unaffected by the different management because its values were similar between sites in the different aggregate fractions.

The values of $\delta^{13}C$ varied from -27.07 and -27.14 ‰ in the oak wood site, and from -27.68 and -27.84 ‰ in the alfalfa site. No specific isotopic fractionation occurred among management, because the $\delta^{13}C$ values did not differ among sites in the different aggregate classes. The $\delta^{15}N$ values, instead, were 0.21‰, 0.24 ‰ and 0.40 ‰ in the oak wood site and 3.39 ‰, 3.64 ‰ and 3.80‰ in the alfalfa site for macro-, meso- and microaggregates respectively. These values showed that alfalfa aggregates were enriched in the heavy N isotope with respected to the oak wood aggregates.

For the mineral phase, considering the main mineral cements (i.e., carbonates and Al and Fe oxides; Bronick and Lal, 2005), the largest part of total Al was in the macroaggregates, which contained 34.68 and 17.95 g kg^{-1} in the oak wood and alfalfa sites, respectively. In the meso- and microaggregates the total Al ranged from 0.98 to 2.77 g kg^{-1}. A similar distribution pattern was observed for total Fe, whose amount in the macroaggregates was 20.37 and 13.16 g kg^{-1} in oak wood and alfalfa sites, while it ranged from 0.59 to 1.68 g kg^{-1} in the meso- and microaggregates. The carbonates content was 85.3, 4.0 and 1.8 g kg^{-1} in the oak wood site and 78.8, 9.6 and 6.6 g kg^{-1} in the alfalfa site for macro-, meso-, and microaggregates respectively. The meso- and microaggregates of the alfalfa site were enriched in carbonates with respect to the oak wood aggregates. The values of pH of soil (before fractionation into aggregates) were similar between

sites, and on the average it was 7.3 for oak wood and 7.6 for alfalfa site.

5.2 Biological parameters

The data on the investigated biological parameters are shown in the Table 5, and, as visible, soil management affected them especially in macro- and mesoaggregates. More in details, macro- and mesoaggregates from oak wood had significant higher values of C_{mic}, C_{extr} and N_{mic}, N_{est} that alfalfa site (C_{mic} amount in macro and mesoaggregates was 280.4, 15.7 mg kg^{-1} and 108.2, 13.9 mg kg^{-1} in oak and alfalfa sites, respectively; C_{extr} was 263.6, 14.2 mg kg^{-1} and 88.9, 8.1 mg kg^{-1}; N_{mic} was 51.6, 2.8 mg kg^{-1} and 14.7, 1.2 mg kg^{-1}; N_{extr} was 106.3, 6.4 mg kg^{-1} and 38.9, 4.4 mg kg^{-1}). These differences were not observed in the microaggregates.

Conversely, in all aggregate classes the C_{mic}/C_{org} and C_{extr}/C_{org} ratios (i.e., the portion of microbial and labile C with respect to the total amount of organic C, respectively) had significantly lower values in oak wood than in alfalfa site. In the oak wood, the C_{mic}/C_{org} values ranged from 4.30 to 6.86, while in alfalfa site from 7.55 to 12.36. The C_{extr}/C_{org} instead ranged from 4.06 to 6.45 and from 5.45 and 10.16 in oak wood and alfalfa site, respectively.

The values of basal microbial respiration (R_{basal}) were high in macroaggregates, especially in oak wood (3.05 and 1.37 µg C-CO_2 $g^{-1}h^{-1}$ respectively in oak and alfalfa macroaggregates). Lower values of R_{basal} were found in meso- and microaggregates, independently from soil management, and ranging from 0.30-0.20 to 0.11-0.15 µg C-CO_2 $g^{-1}h^{-1}$ respectively in oak and alfalfa. The cumulative microbial respiration (R_{cum}) confirmed a certain management effect, having higher values in oak wood than in alfalfa site, especially in meso- and microaggregates, where oak wood had values of 3.29 and 1.84 µg C-CO_2 $g^{-1}28d^{-1}$ in meso- and microaggregates respectively and alfalfa had 2.16 and 1.24 µg C-CO_2 $g^{-1}28d^{-1}$.

Finally, the different management did not influence the values of mineralisation quotient (qM), but the metabolic quotient (qCO_2) had again higher values in meso- and microaggregates of oak wood than in alfalfa site, following thus the same trend of R_{cum}.

Aggregate size fraction	ID	Aggregates mass % (g $100g^{-1}_{soil}$)	C_{org} g kg^{-1}_{soil}	N g kg^{-1}_{soil}	C:N	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	Al_t g kg^{-1}_{soil}	Fe_t g kg^{-1}_{soil}	$CaCO_3$ g kg^{-1}_{soil}
Macro	MO-W	91 a	40.9 a	3.54 a	11.6	-27.13	0.21b	34.68 a	20.37 a	85.3
			(1.9)	*(0.16)*	*(0.02)*	*(0.27)*	*(0.01)*	*(0.03)*	*(0.68)*	*(11.1)*
	MO-A	80 b	8.8 b	0.85 b	10.5	-27.84	3.39 a	17.95 b	13.16 b	78.8
			(0.3)	*(0.16)*	*(1.61)*	*(0.25)*	*(0.17)*	*(0.62)*	*(0.01)*	*(11.2)*
Meso	MO-W	6	2.9	0.24	12.1	-27.07	0.24 b	2.25	1.34 b	4.0 b
			(0.0)	*(0.01)*	*(0.36)*	*(0.17)*	*(0.03)*	*(0.03)*	*(0.05)*	*(1.1)*
	MO-A	11	1.3	0.12	10.8	-27.68	3.64 a	2.77	1.84 a	9.6 a
			(0.3)	*(0.01)*	*(1.72)*	*(0.25)*	*(0.00)*	*(0.71)*	*(0.13)*	*(1.9)*
Micro	MO-W	3	1.3	0.10	12.4	-27.14	0.40 b	0.98	0.59	1.8 b
			(0.6)	*(0.05)*	*(0.46)*	*(0.22)*	*(0.00)*	*(0.50)*	*(0.28)*	*(1.1)*
	MO-A	9	1.2	0.11	10.9	-27.68	3.80 a	2.31	1.68	6.6 a
			(0.7)	*(0.08)*	*(1.05)*	*(0.31)*	*(0.04)*	*(1.46)*	*(1.01)*	*(1.8)*

Table 4. Main chemical characteristics of the aggregate classes. These values ere expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

Aggregate size fraction	ID	C_{mic} mg kg^{-1}_{soil}	N_{mic} mg kg^{-1}_{soil}	C_{mic} /C_{org}	C_{extr} mg kg^{-1}_{soil}	N_{extr} mg kg^{-1}_{soil}	C_{extr}/C_{org}	R_{basal} µg C-CO_2 g^{-1}_{soil} h^{-1}	R_{cum} µg CO_2 g^{-1}_{soil} $28d^{-1}$	qCO_2 µg C-CO_2 $g^{-1}C_{mic}$ h^{-1}	qM µg C- CO_2 $g^{-1}C_{org}$
Macro	MO-W	280.4 a	51.6 a	6.86 b	263.6 a	106.3 a	6.45 b	3.05 a	47.13	1.09	1.14
		(0.8)	*(6.2)*	*(0.31)*	*(4.6)*	*(1.7)*	*(0.20)*	*(0.13)*	*(18.05)*	*(0.09)*	*(0.40)*
	MO-A	108.2 b	14.7 b	12.36 a	88.9 b	38.9 b	10.16 a	1.37 b	10.69	1.26	1.22
		(4.6)	*(0.5)*	*(0.96)*	*(2.5)*	*(2.5)*	*(0.64)*	*(0.05)*	*(1.82)*	*(0.10)*	*(0.26)*
Meso	MO-W	15.7 a	2.8 a	5.37 b	14.2 a	6.4 a	4.86 b	0.30	3.29 a	1.93 a	1.12
		(0.4)	*(0.1)*	*(0.22)*	*(2.8)*	*(0.1)*	*(0.34)*	*(0.00)*	*(0.17)*	*(0.07)*	*(0.09)*
	MO-A	13.9 b	1.2 b	10.46 a	8.1 b	4.4 b	6.12 a	0.20	2.16 b	1.39 b	1.67
		(3.49)	*(0.4)*	*(0.12)*	*(1.7)*	*(0.4)*	*(0.12)*	*(0.06)*	*(0.02)*	*(0.11)*	*(0.31)*
Micro	MO-W	5.6	1.3	4.30 b	5.1	2.1	4.06 b	0.11	1.84 a	1.99 a	0.98
		(2.8)	*(0.5)*	*(0.16)*	*(2.1)*	*(1.0)*	*(0.31)*	*(0.05)*	*(0.15)*	*(0.18)*	*(0.05)*
	MO-A	8.9	1.2	7.55 a	6.3	3.9	5.45 a	0.15	1.24 b	1.71 b	0.80
		(6.1)	*(0.9)*	*(0.35)*	*(4.0)*	*(1.9)*	*(0.08)*	*(0.09)*	*(0.10)*	*(0.19)*	*(0.20)*

Table 5. Main biological characteristics of the aggregate fractions. These values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

5.3 Extracellular enzymatic activities

The macroaggregates of oak wood had higher extracellular enzymatic activities than those of alfalfa site, with the exception of α-glucosidase, which was unaffected by soil management (Table 6). The meso- and microaggregates confirmed that oak wood had higher enzymatic activities related to N, P and S cycle (Table 6). For C cycle oak wood had higher β-glucosidase activity, but α-glucosidase, β-xylosidase and β-cellobiosidase did not differ in meso- and microaggregates between soil management.

		Extracellular enzymatic activity nmol MUF $h^{-1}g^{-1}_{soil}$							
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
Macro	MO-W	432.9 a *(4.5)*	17.5 *(2.9)*	279.3 a *(1.0)*	50.0 a *(5.4)*	73.3 a *(3.4)*	461.7 a *(6.5)*	510.7 a *(13.9)*	200.5 a *(22.4)*
	MO-A	188.4 b *(6.0)*	6.6 *(2.1)*	56.6 b *(9.5)*	22.6 b *(3.1)*	41.9 b *(3.3)*	72.1 b *(2.0)*	101.5 b *(13.3)*	45.9 b *(5.0)*
Meso	MO-W	30.6 a *(3.5)*	1.0 *(0.1)*	19.2 a *(1.9)*	3.2 *(0.4)*	4.5 *(0.0)*	35.9 a *(0.9)*	41.6 a *(0.2)*	12.2 a *(0.5)*
	MO-A	24.7 b *(0.1)*	1.18 *(0.0)*	9.6 b *(3.4)*	4.4 *(0.4)*	6.1 *(0.9)*	11.5 b *(2.5)*	19.4 b *(6.7)*	7.9 b *(2.5)*
Micro	MO-W	18.9 a *(0.5)*	0.5 *(0.3)*	9.3 a *(0.6)*	1.8 *(0.8)*	2.2 *(1.2)*	21.4 a *(0.8)*	23.9 a *(1.4)*	9.4 a *(1.2)*
	MO-A	18.2 b *(0.2)*	1.3 *(0.9)*	6.1 b *(3.0)*	1.44 *(0.5)*	7.64 *(5.2)*	14.5 b *(5.7)*	21.0 b *(0.1)*	6.9 b *(1.9)*

Table 6. Enzymatic activities of the aggregates fraction β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE). These values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at p level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

Tables 7 and 8 shows the enzymatic activities expressed per unit of C_{mic} and C_{org}, thus the enzymatic activities expressed on the basis of different unit of C pools (i.e., microbial and total, respectively). The values related to the labile C (Table 7) confirmed higher enzymatic activity involved in S and P cycle in all aggregate classes of oak wood than alfalfa site. For C and N cycle, different enzymatic behaviour was instead found in the aggregate classes. The C and N enzymatic activities were similar in macroaggregates between oak wood and alfalfa, while they were higher in meso and microaggretas of oak wood site, at least those related to β-glucosidase and N-acetyl β-glucosaminidase.

If we consider the values of enzymatic activity per unit of total organic C, higher S-activity in all aggregate classes was confirmed for oak wood. Higher P-activities was also confirmed, but only in meso- and microaggregates. The C cycle enzymatic activities (β-glucosidase, β-xylosidase and β-cellobiosidase) were instead lower in macro- and meso-aggregates of oak wood site, and no difference was found for α-glucosidase. The N cycle enzymatic activity (N-acetyl β-glucosaminidase) did not differ among soil management.

Extracellular enzymatic activity nmol MUF h^{-1} g^{-1} /mg C_{mic} kg^{-1}									
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
Macro	MO-W	1.54 *(0.01)*	0.06 *(0.01)*	1.00 *(0.00)*	0.18 *(0.02)*	0.26 *(0.01)*	1.65 a *(0.02)*	1.82 a *(0.04)*	0.71 a *(0.08)*
	MO-A	1.74 *(0.13)*	0.06 *(0.02)*	0.53 *(0.11)*	0.21 *(0.04)*	0.39 *(0.05)*	0.67 b *(0.05)*	0.94 b *(0.08)*	0.43 b *(0.06)*
Meso	MO-W	2.21 a *(0.08)*	0.06 *(0.01)*	1.22 a *(0.09)*	0.20 *(0.03)*	0.29 *(0.01)*	2.29 a *(0.01)*	2.65 a (*0.06)*	0.78 a *(0.05)*
	MO-A	1.58 b *(0.05)*	0.09 *(0.02)*	0.68 b *(0.07)*	0.32 *(0.05)*	0.45 *(0.05)*	0.83 b *(0.02)*	1.38 b *(0.15)*	0.57 b *(0.04)*
Micro	MO-W	3.87 a *(0.05)*	0.09 *(0.00)*	1.66 a *(0.03)*	0.62 a *(0.02)*	0.39 *(0.05)*	4.34 a *(0.05)*	6.82 a *(2.19)*	3.35 a *(0.47)*
	MO-A	2.70 b *(0.88)*	0.14 *(0.09)*	1.19 b *(0.28)*	0.44 b *(0.06)*	0.86 *(0.01)*	1.85 b *(0.63)*	3.00 b *(1.82)*	1.13 b *(0.53)*

Table 7. Specific activities of β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE) expressed per unit of C_{mic}. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

Extracellular enzymatic activity nmol MUF h^{-1} g^{-1} /mg C_{org} kg^{-1}									
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
Macro	MO-W	10.59 b	0.43	6.84	1.22 b	1.80 b	11.30 a	12.49	4.92
		(0.40)	*(0.05)*	*(0.30)*	*(0.07)*	*(0.17)*	*(0.38)*	*(0.26)*	*(0.78)*
	MO-A	21.49 a	0.75	6.44	2.58 a	4.77 a	8.22 b	11.62	5.23
		(0.06)	*(0.21)*	*(0.86)*	*(0.27)*	*(0.21)*	*(0.06)*	*(1.92)*	*(0.39)*
Meso	MO-W	8.46 b	0.34	6.57	1.09 b	1.54 b	12.27 a	18.24 a	4.16
		(0.07)	*(0.01)*	*(0.73)*	*(0.11)*	*(0.02)*	*(0.46)*	*(0.23)*	*(0.13)*
	MO-A	23.09 a	0.91	7.14	3.35 a	4.67 a	8.66 b	14.44 b	5.93
		(0.57)	*(0.23)*	*(0.86)*	*(0.47)*	*(0.43)*	*(0.15)*	*(1.69)*	*(0.48)*
Micro	MO-W	16.47	0.40	7.15	1.37	1.68 b	18.49 a	20.55 a	7.76 a
		(8.16)	*(0.03)*	*(0.16)*	*(0.03)*	*(0.13)*	*(3.10)*	*(2.61)*	*(1.80)*
	MO-A	15.07	1.04	8.91	4.27	6.52 a	13.84 b	14.31 b	5.92 b
		(6.21)	*(0.16)*	*(3.17)*	*(0.0)*	*(0.24)*	*(2.09)*	*(1.41)*	*(0.05)*

Table 8. Specific activities of β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE) expressed per unit of C_{org}. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

5.4 Total DNA and fungi diversity

The amounts of total extracted DNA (DNA_{tot}) showed higher values in the oak wood site than alfalfa site; however differences were not statistically significant due to high variability of extracted DNA from the two sites (Table 9). In particular, in all aggregate fractions of oak wood we found higher values in the second replication compared to the first (the values of extracted DNA ranged from 0.14 to 4.08 g μg^{-1} for the first replication and from 0.46 to 7.63 g μg^{-1} for the second replication). Alfalfa site also showed the same pattern with the exception of macroaggregates.

In both sites, the DNA_{tot}/C_{mic} ratio had high values in microaggregates, and in oak wood it was significantly higher than in alfalfa (DNA_{tot}/C_{mic}: 0.04, 0.08 and 0.05, 0.06 μg^{-1}soil in the 1° and 2° of oak wood and alfalfa sites, respectively). In the macro- and mesoaggregates the DNA_{tot}/C_{mic} ranged from 0.01 to 0.05 g μg^{-1}soil, and no differences between soil management were found.

For the mesoaggregates the Shannon-Wiener index (H') was lower in oak wood than in alfalfa site (0.63, 0.57 and 0.84, 0.83 in the 1° and 2° of oak wood and alfalfa sites, respectively). Alfalfa mesoaggregates thus showed greater microbial diversity than oak wood mesoaggregates. In the macro- and microaggregates the Shannon-Wiener index had high variability, ranging from 0.34 to 0.88, and no further differences were found between soil management. The Eveness, or equitability, measured by Pielou's index (J'), did not change between sites in all aggregate fractions.

Aggregate size fraction	ID	Sampling	DNA_{tot} g μg^{-1}_{soil}	DNA_{tot}/C_{mic} g μg^{-1}_{Cmic}	*H'*	*J'*
Macro	MO-W	1	4.08 *(0.6)*	0.01	0.63	0.92
		2	7.63 *(0.9)*	0.03	0.34	0.72
	MO-A	1	3.03 *(0.6)*	0.03	0.78	0.85
		2	1.46 *(0.5)*	0.01	0.63	0.81
Meso	MO-W	1	0.21 *(0.1)*	0.01	0.63 b	0.90
		2	0.46 *(0.1)*	0.03	0.57 b	0.78
	MO-A	1	0.45 *(0.1)*	0.03	0.84 a	0.95
		2	0.52 *(0.2)*	0.05	0.83 a	0.86
Micro	MO-W	1	0.14 *(0.0)*	0.04 a	0.88	0.87
		2	0.63 *(0.1)*	0.08 a	0.40	0.58
	MO-A	1	0.22 *(0.1)*	0.05 b	0.72	0.82
		2	0.80 *(0.2)*	0.06 b	0.73	0.94

Table 9. Amounts of DNA extraction efficiency (DNA_{tot}) total DNA/C_{mic} ratio, Shannon Wiener (H') and Eveness (J') index in aggregate fractions. These values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The number 1 and 2 indicate the two field replications on each sites (1° sampling and 2° sampling).

The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

The effect of the different management on the clustering analysis of the DGGE patterns of all aggregate fractions was reported in Fig 5. Cluster analysis of macroaggregates (Fig 5a) indicates a first division between the oak wood macroaggregates and macroaggregates of alfalfa (similarity <35%). A second clustering level (similarity <60%) separated the two field replications within the same site. In particular, the first sampling (MO-A 1) from the second alfalfa site (MO-A 2) and also for the oak wood site (MO-W 1 separated by MO-W2).

The same behaviour was observed for the microaggregates cluster analysis (Fig. 5c) that showed a first division between oak wood microaggregates and microaggregates of alfalfa (similarity <35 %). A second clustering level separated the two field replications within oak wood site, (MO-W 1 and MO-W 2), with a similarity less than 45%. The third separation was observed within the alfalfa site between field replications, MO-A1 and MO-A2 (similarity <60%).

In the cluster analysis of mesoaggregates (Fig 5b) a first division was observed among all field replications of alfalfa (MO-A1 and MO-A2) and the second sampling of the oak wood (MO-W2) from the first sampling of the oak wood (MO-W1), with a similarity <20%. A second clustering level separated the field replications of the alfalfa (MO-A1 and MO-A2) from the second of oak wood (MO-W2), with similarity less than 30%.

Similarity among field replicates was always higher than 70 % for all aggregates.

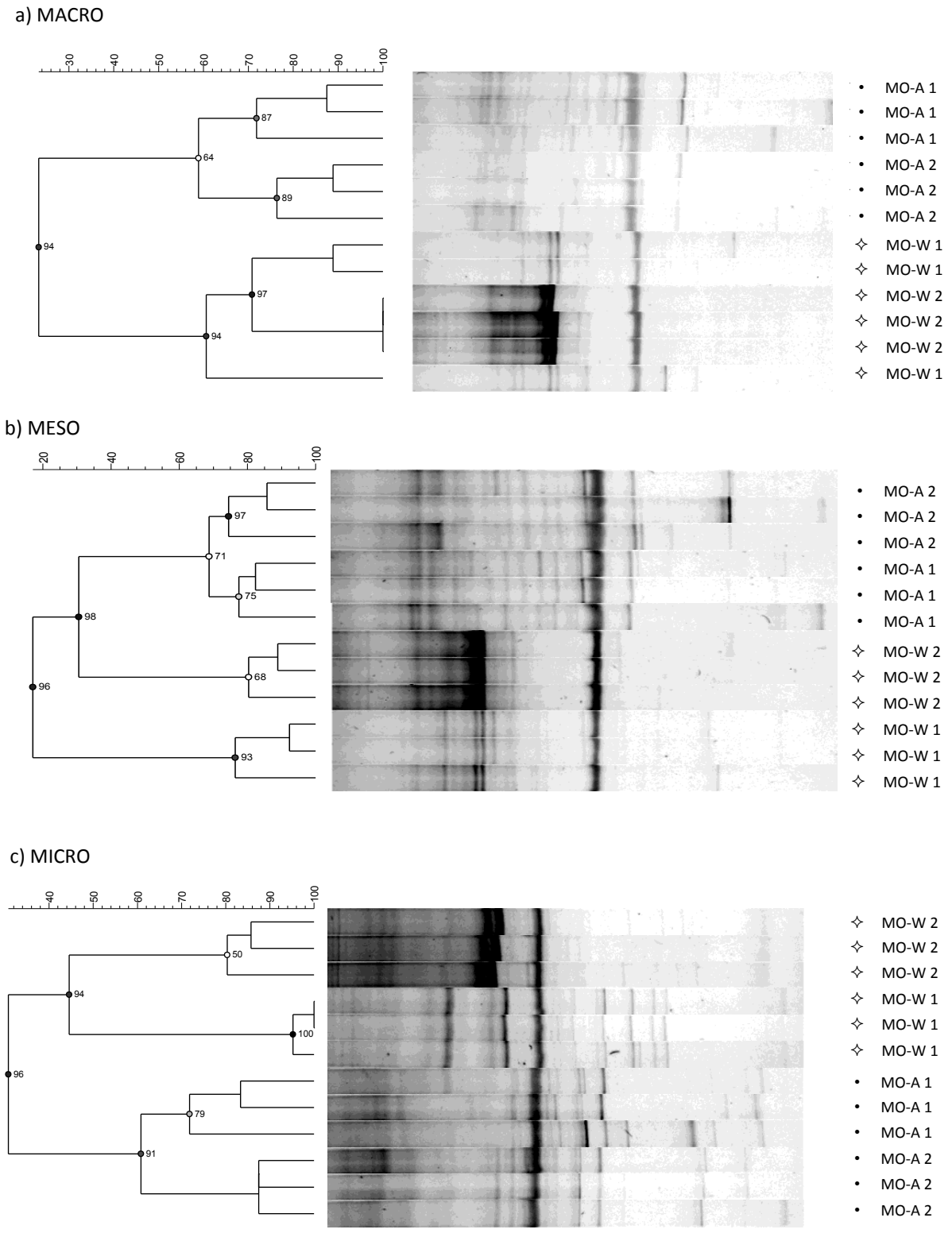


Figure 5. Effect of the different management on the clustering analysis of the DGGE patterns of macroaggregates (5.a) mesoaggregates (5.b) and microaggregates (5.c). MO-A and MO-W indicate alfalfa and oak wood sites and the number 1 and 2 indicate the two field replications in each sites (1° sampling and 2° sampling).

5.5 Physical parameters

The physical parameters of aggregates are shown in Table 10. For particle size distribution the data of clay and sand, both as total sand (i.e., particle size between 2 and 0.05 mm) and coarse sand (i.e., particle size between 2 and 0.2 mm), has been reported, stressing thus the differences related to contrasting particle size (fine and coarse particle, respectively). The macroaggregates of oak wood were more enriched in clay and coarse sand particles than those of alfalfa site (clay: 39.1 g kg^{-1} and 12.9 g kg^{-1} in oak wood and alfalfa, respectively; coarse sand: 18.8 g kg^{-1} and 4.6 g kg^{-1}, respectively). The differences in coarse sand were also observed in the meso- and microaggregates, but in this case, the coarse sand was more abundant in the alfalfa aggregates.

Significant differences were also found in both the total pore volume (V_{Hgtot}) and pore size distribution. The V_{Hgtot} was always higher in oak wood aggregates than in alfalfa (in macroaggregates 156.82 *vs.* 101.59 mm^3 g^{-1}, in mesoaggregates 22.83 *vs.* 12.89 mm^3 g^{-1}, in microaggregates 1.04 *vs.* 5.30 mm^3 g^{-1}; Table 10). In each aggregate class, at higher total pore volume corresponded higher specific surface area of the pore (SSA_{tot}; 7.63, 0.51 and 0.32 m^2 g^{-1} in oak wood site, and 1.51,0.38 and 0.26 m^2 g^{-1} in alfalfa site).

As regards to the pore size distribution, in all the aggregate classes the pores had a unimodal distribution (Fig 6). Independently from soil management, the pores of 1-0.05 μm were the most frequent in macroaggregates. In meso- and microaggregates, pores of 25-1 and 1-0.05 μm were instead the most frequent. In the macroaggregates, the volume of pores <1 μm (1-0.05 and <0.05 μm) was higher in oak wood site than in alfalfa site. The <1 μm pores represented the 70 and 54% of the total pore volume in oak and alfalfa, respectively; therefore the higher porosity of oak-macroaggregates appeared ascribable to very small pores. In mesoaggregates the pore size distribution was similar among soil management, with the exception of a slight higher presence of 75-50 μm pores in alfalfa. This class of pores was however only 7 and 11% of the total pore volume in oak wood and alfalfa site; thus the higher porosity of oak-mesoaggregates was not due to a specific pore class, but it was distributed among the other pore size classes (<50 μm). In microaggregate, the largest pore classes (75-50 and 50-25 μm) were not detected. The other pore size classes had always significant higher volume in oak than in alfalfa (Fig 6), and thus the higher porosity of oak was due to higher presence of all pore size classes (<25 μm).

Aggregate size fraction	ID	Clay (<2 µm) g kg^{-1}	Total sand (2-0.05 mm) g kg^{-1}	Coarse Sand (2-0.2 mm) g kg^{-1}	V_{Hgtot} mm^3 g^{-1}	SSA_{tot} m^2g^{-1}
Macro	MO-W	39.1 a	34.6	18.8 a	156.82 a	7.63 a
		(2.1)	*(2.8)*	*(2.0)*	*(14.32)*	*(0.36)*
	MO-A	12.9 b	40.9	4.6 b	101.59 b	1.51 b
		(4.6)	*(1.4)*	*(0.1)*	*(3.63)*	*(1.16)*
Meso	MO-W	2.0	1.9	0.3 b	22.83 a	0.51 a
		(0.2)	*(0.3)*	*(0.1)*	*(4.98)*	*(0.15)*
	MO-A	2.8	5.72	2.7 a	12.89 b	0.38 b
		(0.2)	*(1.6)*	*(0.3)*	*(1.04)*	*(0.05)*
Micro	MO-W	1.0	0.49	0.1 b	11.04 a	0.32 a
		(0.5)	*(0.14)*	*(0.0)*	*(3.72)*	*(0.02)*
	MO-A	2.3	3.1	0.4 a	5.30 b	0.26 b
		(1.7)	*(1.5)*	*(0.1)*	*(2.62)*	*(0.02)*

Table 10. Main physical characteristics of aggregate fractions. These values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. Total sand: coarse+fine sand; V_{Hgtot}: total volume of pores; SSA_{tot}: specific surface area of the pores. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

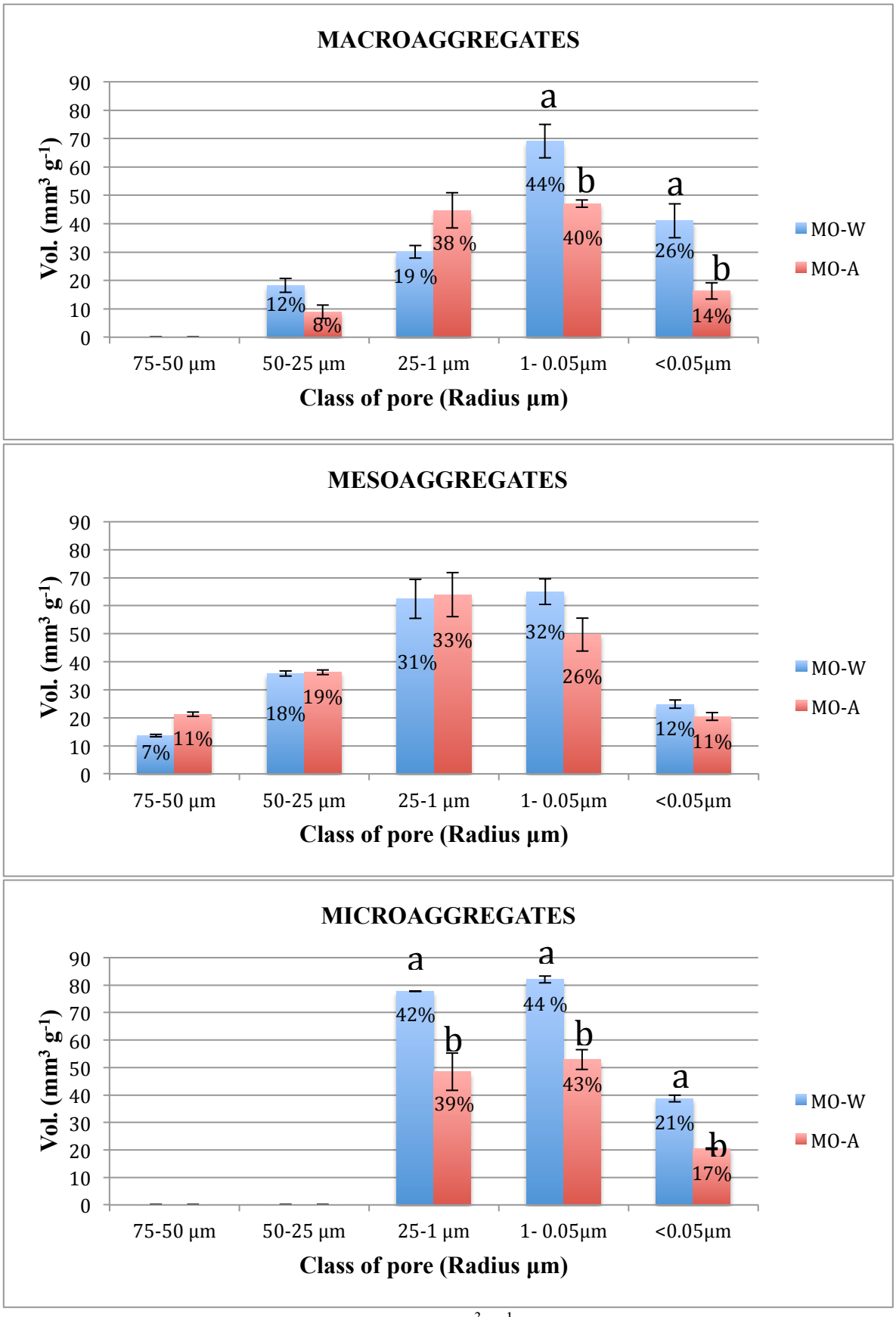


Figure 6. Pore size distribution expressed as mm^3 g^{-1}soil. The numbers inside the graph show the pore size distribution expressed as percentage [(volume of pore class/V_{HgTot})·100]. The letters show the significant differences at *p* level <0.05 Tukey test among oak wood and alfalfa sites in macro-, meso- and microaggregates

5.6 *Discussion*

Soil management effects on macroaggregates

Macroaggregates represented more than 80% of total soil mass in both sites. The aggregate size distribution thus demonstrated that soil aggregation enhanced macroaggregate formation over other smaller aggregate classes. This was in agreement with Golchin et al., (1994), Jastrow (1996) and Six et al., (1999a) where fresh residue induced the formation of macroaggregates as being a C source for microbial activity and the production of microbial-derived binding agents. As expected, the soil organic carbon was mainly contained in the macroaggregates, and the greatest amount of C_{org} was found in the oak wood macroaggregates. This could be the result of both larger organic input and reduced microbial decomposition in an oak wood compared to alfalfa (Yanni et al., 2011). A large organic input of plant residues was expected in an oak wood as arboreous species produce more biomass production than herbaceous species. Moreover it was expected that oak residues would decompose less easily than alfalfa. This was because the soil organic matter of oak wood would be richer in lignin than in the alfalfa site and hence more difficult to degrade. The lignin content of residues was one of the main factors affecting decomposition due to the recalcitrance of this complex molecule and its resistance to degradation by soil microorganisms and extracellular enzymes (Austin and Ballare, 2010; Cadisch and Giller, 1997; Melillo et al., 1982). Residues with high lignin content were expected to decompose more slowly, and persist longer in soils than residues with low lignin content. On the other hand, crop residues with high N concentrations, low C/N ratios and low lignin concentrations, such as those derived from legumes like alfalfa, tend to decompose faster (Chivenge et al., 2011; Yanni et al., 2011). This was also confirmed by Min et al (2003), who reported that alluvium soils under alfalfa have high soil aggregation induced by higher crop root mass and easy litter decomposition.

The biological properties of our macroaggregates indicated a higher rate of microbial basal respiration (R_{basal}) in the oak wood, coupled whit similar values of microbial cumulative respiration (R_{cum}) and C mineralization quotient (qM) between sites. From these results it seems confirmed that the larger C input from oak residues increases basal respiration, due to more recalcitrant organic molecules, but this is counterbalanced by the very efficiency with which microorganisms metabolize organic matter from alfalfa residues. This may again be due to the different quality of the soil organic matter between the oak wood and alfalfa sites, and the consequently different availability of C input for microorganisms. Macroaggregates were generally considered to be dominated by fungi (Frey, 2005), but we can probably rule out the differences in biological

properties being due to any differences in fungi population because, even if the DGGE pattern indicated a low similarity between sites (<30%), the amount of fungi DNA extracted was similar.

The C pool data seem to confirm lower substrate availability in macroaggregates from an oak wood. In fact, although a higher value of microbial biomass (C_{mic}) was found in oak wood macroaggregates than in alfalfa, the microbial biomass reduced more rapidly than the organic matter content, as shown by the lower C_{mic}/C_{org} ratio value in the oak wood site. The C_{mic}/C_{org} ratio, named 'microbial quotient' by Sparling (1992), is in fact an indicator of biological activity and accumulation of organic matter in soil. Low values indicate that the biotope is not tending establish energetic metabolism on the part of microorganisms. Anderson and Domsch (1989) report that the microbial quotient also reflects the C substrate availability for soil microorganisms. Thus, the observed low value in oak wood macroaggregates may indicate lower substrate availability for soil microorganisms. On the other hand the lower microbial quotient in oak corresponded to a similar level of microbial biomass specific activity (qCO_2) between soil management, thus suggesting that the microbial population in the oak wood seemed to have adapted to this poorly available C resource. In the oak wood site the quality and composition of the litter may therefore negatively affect the substrate availability for microbial biomass, which responds to this stress by increasing its activity (Allison et al., 2010).

Adaptation on the part of microbial activity in the oak wood was also confirmed by values of enzyme activity, which were generally higher there than in the alfalfa site. The α-glucosidase activity was an exception, since it did not differ between sites, but it was related to the decomposition of starch molecules, which degradable more easy than other compounds in the C cycle. The enzyme activities expressed on the basis of microbial biomass enable one to assess the metabolic status of the microbial community. Coombining the information obtained from qCO_2 (Landi et al., 2000) and the activity related to the carbon and nitrogen cycle (β-glucosidase, N-acetyl β-glucosaminidase, β-xylosidase, β-cellobiosidase), it was observed that they reflected the same metabolic status for both sites. By contrast, arylsulfatase, phosphomonoesterase and phosphodiesterase activities showed higher metabolic status in the oak wood than in alfalfa. The specific enzyme activities confirmed a lower substrate availability under oak than under alfalfa either due to adaptation by the microbial population (as for S and P enzyme activities) or due to a less efficient system (as for C and N enzyme activities).

It was, however, possible that the different quantity and quality of organic matter input, due to soil managements, might make it difficult to compare the absolute values of investigated enzyme activities between sites, thus foiling any clear diagnosis of the effect of management on soil quality. One way of overcoming this difficulty and salving comparison of oak wood and alfalfa sites was to use the values of specific activity per unit of carbon (Barriuso et al., 1988). Values of specific activities per carbon unit revealed that in macroaggregates the N-acetyl β-glucosaminidase, α-glucosidase, phosphomonoesterase and phosphodiesterase activity were not affected by organic C availability because they were similar in both sites. By contrast, arylsulfatase and the C cycle enzyme (β-glucosidase, β-xylosidase, β-cellobiosidase) changed between different management. In particular, arylsulfatase activity showed higher values in the oak wood than in alfalfa while for the C cycle enzymes (β-glucosidase, β-xylosidase, β-cellobiosidase) we found lower values. The opposite trend with S and C cycle enzymes may be due to the large amount of cellulose material added to the alfalfa sites, as indicated by the trend of activity per unit of microbial biomass.

The different pore system between sites could be another factor affecting organic matter dynamics in macroaggregates. In the oak wood we found a more porous system characterized by a predominance of very fine pores (1-0.05 μm) which corresponded to textural pores originating from clay-clay interactions and therefore related to the amount of clay particles (Attou and Le Bissonnais, 1998). The presence of small pores would lead to physical protection of organic matter, a further reason why microbial distribution might be limited in an oak wood. (Zaffar and Lu., 2015)

Soil management effects on meso- and microaggregates

Meso- and microaggregates, which represented less than 20% of the soil mass in both sites, did not differ between oak wood and alfalfa in the amount of organic C. The similarity in C content -not found in macroaggregates- may have been due to transformation of the organic matter during the aggregation process. In the "aggregate hierarchy" hypothesis, it is postulated that soil organic carbon concentration declines with decreasing aggregate size (Dexter, 1988; Tisdall and Oades, 1982) but that organic carbon in small aggregates is more stable and resistant to degradation. This stabilization of organic carbon in soil aggregates is due mainly to the aggregate architecture (i.e., physical occlusion) and the protection of organic carbon from microbial decomposition through formation of clay–organic carbon complexes (Sollins et al., 1996). It could thus well be that, in the absence of dissimilarities among other environmental factors besides soil organic matter quantity and quality input, the aggregation process expresses its maximum effect on meso- and microaggregates, limiting the differences due to soil management whisch are visible in

macroaggregates. This is in agreement with the well-known different effect of management on macro- and microaggregates mentioned by von Lützow et al., (2006), who stated that microaggregates were not affected by management.

In mesoaggregates we found the same values of microbial basal respiration (BR_{basal}) and C mineralization quotient (qM) between oak and alfalfa, but a higher value of cumulative respiration (BR_{cum}) in the oak wood site. C in the microbial biomass was also higher in the oak wood, but when it was expressed as a C_{mic} to C_{org} ratio (i.e. microbial quotient reflecting the availability of C substrates for soil microorganisms), it was lower in oak than in alfalfa. Thus the microbial quotient showed a similar trend to macroaggregates, confirming that the oak wood site established limited energetic metabolism on the part of many microorganisms and produced low substrate availability for soil microorganisms, which was also confirmed by the higher BR_{cum} value.
In alfalfa sites, on the other hand , the larger microbial quotient (C_{mic}/C_{org}) corresponded to a very low level of specific microbial biomass (qCO_2) and enzyme activity compared to the oak wood site. This indicated a predominance of non metabolically active microorganisms and/or of more efficient organisms. This may have been caused by the diverse nature and quality of the organic substrate (Nsabimana et al., 2004), which was more available in alfalfa. Microbial production of extracellular enzymes is in fact affected by substrate availability, but also by microbial community. Changes in extracellular enzyme activity may also be due to shifts in microbial community membership, particularly fungi (Kaiser et al., 2010). This last hypothesis was further confirmed by the Shannon-Winer index which showed that the alfalfa site had a higher fungal microbial diversity.

As was observed in macroaggregates, in mesoaggregates enzyme activities values were generally higher in the oak wood than in alfalfa sites, with the exception of α-glucosidase, β-xylosidase and β-cellobiosidase. Thus all the enzymatic activities were higher in the oak wood, with the exception of those related to the most labile investigated C compounds (i.e., starch and hemicelluloses; Donovan et al., 2012). This difference vis-à-vis macroaggregates (i.e., the lack of difference in β-xylosidase and β-cellobiosidase activities) would be a first sign of the presence of more decomposed organic matter in mesoaggregates than in macroaggregates. The hemicelluloses are a group of polysaccharides of differing composition, which consist of cellulose-like sugar units, bound together with glycosidic linkages, though more or less strongly branched and having a lower degree of polymerization than cellulose. Their decomposition rate is higher than that of cellulose (Swift et al., 1979). Enzyme activities expressed per unit of microbial biomass allowed us to detect the same trend for α-glucosidase, β-xylosidase and β-cellobiosidase in the two sites confirming the same

metabolic status in both sites for labile and moderately decomposable C compounds. Again, enzyme activities such as β-glucosidase, arylsulfatase, P- cycle activities and N-acetyl β-glucosaminidase showed a higher metabolic status in the oak wood than in alfalfa. These differences could be explained if we hypothesize that organic matter in mesoaggregates was more degrades more than in macroaggregates.

The specific enzyme activities per unit of organic carbon revealed that N-acetyl β-glucosaminidase, α-glucosidase and phosphodiesterase were not affected by organic C availability, being similar between sites. One the other hand, arylsulfatase, phosphomonoesterase and the C cycle enzymes (β-glucosidase, β-xylosidase, β-cellobiosidase) showed differences between sites. In particular, arysulfatase and phosphomonoesterase activities had higher values in the oak wood than in alfalfa, while for the C cycle enzymes (β-glucosidase, β-xylosidase, β-cellobiosidase) we found lower values in the oak wood than in alfalfa. However, one cannot rule out an effect by the different particle size distribution in mesoaggregates, and in particular the great amount of coarse sand in alfalfa. In general, very high carbohydrases (β-glucosidase, β-xylosidase, β-cellobiosidase) have been measured in coarse sand (Stemmer et al., 1998; Kandeler et al., 1999a; Marx et al., 2005) probably due to greater soil enzyme absorption to less mineralized particulate organic matter which is often related to a coarser fraction (Stemmer et al., 1998).

Pore systems did not seem to affect the C dynamics, aswe found the oak wood to be a more porous system characterized by large pores (25-1 μm). This should actually favour the microbial activity (Lèo S. et al 2013), enhancing organic matter decomposition, but a limitation of microbial activity rather than an enhancement occurred in oak mesoaggregates. The C dynamics thus seemed more affected by the aggregate formation process and presence of more degraded organic compounds, as the biological and biochemical parameters had showen.

Microaggregates reflected the same conditions as observed in mesoaggregates, with the exception of enzyme activity values expressed per unit of organic C. β-glucosidase and β-xylosidase activities were similar in microaggregates from both sites, while a significant difference was found for phosphodiesterase activity which was not observed in mesoaggregates. This datum further confirmed the hypothesis that aggregate formation produces smaller aggregates enriched with transformed organic molecules. It is important to note that even in the microaggregate oak wood site we found a higher pore system characterized by large pores. Strong et al. (2004), using a correlative approach, suggested that pores with a radius <30 contained more nematodes and more

fungal biomass, but less total biomass. This was in agreement with the higher DNA values expressed on microbial carbon which we found to be significantly higher in the oak wood that in alfalfa.

5.7 *Conclusions*

The multidisciplinary approach used enabled us to assess the different effects of the oak wood and alfalfa sites on biophysical properties, and therefore on the quality of soil. In particular, in the macroaggregate fraction the biophysical properties of the oak wood revealed a system that did not promote microbial activity (i.e., specific activities per unit of carbon) despite its great input of organic C. This was mainly due to the quality of the organic matter and the physical architecture of the aggregates affected by their texture. In macroaggregates from the oak wood we found a more porous system, but characterized by a predominance of very fine pores, such as physical protection of organic matter, a further reason that may have limited SOM availability for the microbial population. The lesser availability of oak organic matter was confirmed by biological parameters (such as the microbial quotient and qCO_2) and by the values of enzymatic activity. In the oak wood site the quality and composition of the litter thus adversely affected substrate availability for microbial biomass, which had responded to this stress by stepping up activity. Conversely, in mesoaggregates from the alfalfa site we found a predominance of non metabolically active microorganisms and/or more efficient organisms that may have been induced by the diverse nature and quality of the organic substrate

In meso- and microaggregates, the C dynamics seemed more affected by the aggregate formation process and the presence of more degraded organic compounds, as biological and biochemical parameters had showen. The high degradation of organic matter in these aggregates may have been due to the genesis of aggregates, since the first step for genesis of small aggregates within macroaggregates comes from the process of degradation of organic matter (Six et al., 1999b).

Biophysical investigation also provided some interesting information on the different aggregate fraction in response to soil management. In macroaggregates one could attribute the low efficiency observed in the oak wood site to the C input (presence of lignin) and, subsequently, to the different particle size distribution in this aggregate fraction. By contrast, the study of meso- and microaggregates confirmed that aggregate formation produces smaller aggregates enriched with transformed organic molecules, and found that mesoaggregates (i.e. therefore between 1 mm and 250 μm) behaviour was more similar to microaggregates than macroaggregates (and hence much

more influenced by the aggregative process than by management). According to Tisdall and Oades (1982), microaggregates (<250 μm) are little influenced by management, but according to our study this aggregate size limit (of 250 μm) should be moved to at least 1 mm, at any rate when the management does not have a strong influence on aggregation as in our oak wood and alfalfa sites.

6. NITROGEN FERTILIZATION EFFECT ON BIOPHYSICAL PROPERTY OF DIFFERENT AGGREGATES FRACTIONS (MICROHABITATS) IN A PLAIN AGROECOSYSTEM.

6.1 Aggregate size distribution and chemical parameters

In the walnut control site the macro-, meso-, microaggregates accounted for the 60, 18 and 22% of the total mass of soil, respectively (Table 11). In the walnut fertirrigate site they accounted for the 70, 19 and 11% and for the walnut granular site they accounted for the 71, 11 and 18%. The macroaggregates represented in all sites the largest part of the mass of aggregates, and their amount was similar between sites.

In all sites, the largest part of soil organic C was in the macroaggregates (Table 11), which contained 3.9, 5.5 and 6.3 g kg^{-1} in the walnut control, fertirrigate and granular sites, respectively. In the meso- and microaggregates the organic C ranged from 0.9 to 1.7 g kg^{-1}. A similar distribution pattern was observed for total N, whose amount in the macroaggregates was 0.49, 0.67 and 0.72 g kg^{-1} for walnut control, fertirrigate and granular sites respectively, while it ranged from 0.11 to 0.19 g kg^{-1} in the meso- and microaggregates. The organic C to nitrogen ratio (C/N) varied from 7.97 and 8.66 in the walnut control site, from 8.23 and 8.92 in the walnut fertirrigate site and from 8.54 and 8.68 in the walnut granular. The C/N was unaffected by different management because its values were similar between sites in the different aggregate fractions.

The $\delta^{13}C$ values were -24.81‰, -24.78 ‰ and -24.75 ‰ in the macro-, meso- and microaggregates in walnut control site. The $\delta^{13}C$ values were -25.99‰, -25.78 ‰ and -25.75 ‰ in the walnut fertirrigate site and were -25.98‰, -25.81‰ and -24.53 ‰ in the walnut granular for macro-, meso- and microaggregates respectively. This data showed that walnut control aggregates (especially in macroaggregates) were enriched in the heavy C isotope with respect to the walnut aggregates characterized by the addition of urea. The $\delta^{15}N$ varied from 7.29 and 7.60‰ in the walnut control, from 6.37 and 6.87‰ in the walnut fertirrigate site and from 6.79 and 7.04‰ in the walnut granular. No specific N isotopic fractionation occurred among management, because the $\delta^{15}N$ did not differ among sites in the different aggregate classes.

For the mineral phase, considering the main mineral cements (i.e., carbonates and Al and Fe oxides; Bronick and Lal, 2005), the largest part of total Al was in the macroaggregates, which contained 16.82, 23.27 and 23.37 g kg^{-1} in the walnut control, fertirrigate and granular sites, respectively. In the meso- and microaggregates the total Al ranged from 3.16 to 6.35 g kg^{-1}. A similar distribution

pattern was observed for total Fe, whose amount in the macroaggregates was 12.60, 15.93 and 16.51 g kg^{-1} in the walnut control, fertirrigate and granular sites, while it ranged from 2.36 to 4.40 g kg^{-1} in the meso- and microaggregates. No differences were found in Al and Fe total content among sites. Walnut control, fertirrigate and granular aggregates were characterized by the absence of carbonates and the pH values (before fractionation into aggregates) was similar among sites, and it was 6.6 for walnut control, 5.9 for walnut fertirrigate and 6.3 for walnut granular.

6.2 Biological parameters

The data on the investigated biological parameters are shown in the Table 12. More in details, macroaggregate from walnut fertirrigate and walnut granular sites had significant higher values of C_{mic} and C_{extr} than walnut control site (C_{mic} amount in macroaggregates was 50.6, 52.2 and 29.8 mg kg^{-1} in walnut fertirrigate, granular and control sites, respectively; C_{extr} was 72.7, 75.8, 68.3 mg kg^{-1}). In all sites, the largest part of N_{mic} and N_{extr} were in the macroaggregates, which contained 3.6, 7.0 and 7.1 mg kg^{-1} of N_{mic} in the walnut control, fertirrigate and granular and 10.2, 10.3 and 11.6 mg kg^{-1} for N_{extr} respectively. In the meso- and microaggregates the N_{mic} ranged from 0.7 to 1.8 mg kg^{-1} and N_{extr} was 1.9 to 2.8 mg kg^{-1}.

In macroaggregate the C_{extr}/C_{org} ratio, that represented the labile C portion with respect the total amount of organic C, had significant lower values in walnut fertirrigate and granular than in walnut control site (13.37 in the walnut fertirrigate, 12.41 in the walnut granular and 17.88 in the walnut control). These differences were not observed for the C_{mic}/C_{org} ratio, which range from 7.76 to 9.25.

In the meso- and microaggregates, the ratio of C_{extr}/C_{org} ranged from 10.40 to 12.95, and the C_{mic}/C_{org} values ranged from 5.02 to 8.35. Both ratios did not differ among sites.

The values of microbial basal respiration (R_{basal}) were low in the meso- and microaggregates (0.01 μg $C\text{-}CO_2$ $g^{-1}h^{-1}$), independently from soil management. Greater values of R_{basal} were instead found in macroaggregates, especially in walnut granular with respect to walnut control and fertirrigate (0.07, 0.04 and 0.04 μg $C\text{-}CO_2$ g^{-1} h^{-1}, respectively). The cumulative microbial respiration (R_{cum}) confirmed a certain effect of management, having higher values in walnut granular than in walnut control and fertirrigate sites, especially in macro- and microaggregates. R_{cum} amount in macroaggregates was 2.88, 1.05 and 1.89 μg $C\text{-}CO_2$ g^{-1} 15 d^{-1} in walnut granular, control and fertirrigate sites, respectively; R_{cum} in microaggregates 0.61, 0.37 and 0.32 μg $C\text{-}CO_2$ g^{-1} 15 d^{-1}, respectively. These differences were not observed in the mesoaggregates. Finally, in macro-, meso- and microaggregates the qM values ranged from 0.26 to 0.54 mg μg $C\text{-}CO_2$ $g^{-1}C_{org}$ 15 $d^{-1}$and the

qCO_2 values ranged from 0.9 to 1.7 μg C-CO_2 $g^{-1}C_{mic}$ h^{-1}. The different management influenced neither the qM, nor the qCO_2 values.

Aggregate size fraction	ID	Aggregate mass % (g $100g^{-1}_{soil}$)	C_{org} g kg^{-1}_{soil}	N g kg^{-1}_{soil}	C:N	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	Al_t g kg^{-1}_{soil}	Fe_t g kg^{-1}_{soil}	$CaCO_3$ g kg^{-1}_{soil}
Macro	PL-CONT	60	3.9	0.49	7.97	-24.81 a	7.29	16.82	12.60	0
			(0.6)	*(0.04)*	*(0.62)*	*(0.17)*	*(0.57)*	*(3.42)*	*(2.53)*	
	PL-FERT	70	5.5	0.67	8.23	-25.99 b	6.37	23.27	15.93	0
			(0.7)	*(0.06)*	*(0.31)*	*(0.30)*	*(0.22)*	*(2.71)*	*(0.67)*	
	PL-GRAN	71	6.3	0.72	8.68	-25.98 b	7.04	23.37	16.51	0
			(1.5)	*(0.11)*	*(0.80)*	*(0.12)*	*(0.25)*	*(6.93)*	*(3.28)*	
Meso	PL-CONT	18	1.4	0.16	8.66	-24.78	7.33	5.55	3.97	0
			(0.2)	*(0.01)*	*(0.13)*	*(0.17)*	*(0.20)*	*(1.14)*	*(0.62)*	
	PL-FERT	19	1.7	0.19	8.92	-25.78	6.87	6.35	4.40	0
			(0.4)	*(0.02)*	*(0.99)*	*(0.35)*	*(0.52)*	*(1.41)*	*(0.67)*	
	PL-GRAN	11	0.9	0.11	8.63	-25.81	6.83	3.16	2.47	0
			(0.3)	*(0.04)*	*(0.75)*	*(0.13)*	*(0.12)*	*(0.26)*	*(0.56)*	
Micro	PL-CONT	22	1.6	0.19	8.17	-24.75	7.60	5.78	4.26	0
			(0.7)	*(0.08)*	*(0.22)*	*(0.22)*	*(0.53)*	*(0.63)*	*(0.95)*	
	PL-FERT	11	1.0	0.11	8.38	-25.75	6.86	3.55	2.36	0
			(0.6)	*(0.06)*	*(0.58)*	*(0.00)*	*(0.16)*	*(1.72)*	*(0.92)*	
	PL-GRAN	18	1.5	0.18	8.54	-25.53	6.79	5.25	3.86	0
			(0.1)	*(0.02)*	*(0.41)*	*(0.14)*	*(0.46)*	*(1.65)*	*(0.54)*	

Table 11. Main chemical characteristics of the aggregate fractions. These values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. The numbers in parentheses and italic are standard deviation values.

Aggregate size fraction	ID	C_{mic} mg kg^{-1}_{soil}	N_{mic} mg kg^{-1}_{soil}	C_{mic} /C_{org}	C_{extr} mg kg^{-1}_{soil}	N_{est} mg kg^{-1}_{soil}	C_{extr} /C_{org}	R_{basal} µg C-CO_2 g^{-1}_{soil} h^{-1}	R_{cum} µg C-CO_2 g^{-1}_{soil} $15d^{-1}$	qCO_2 µg C-CO_2 $g^{-1}C_{mic}$ h^{-1}	qM µg C-CO_2 $g^{-1}C_{org}$
Macro	PL-CONT	29.8 b	3.6	7.76	68.3 b	10.2	17.88 a	0.04 b	1.05 b	1.24	0.30
		(0.8)	*(1.6)*	*(0.99)*	*(4.7)*	*(2.8)*	*(4.02)*	*(0.01)*	*(0.40)*	*(0.41)*	*(0.15)*
	PL-FERT	50.6 a	7.0	9.25	72.7 a	10.3	13.37 b	0.04 b	1.89 b	0.88	0.34
		(2.1)	*(0.5)*	*(0.78)*	*(3.7)*	*(0.4)*	*(2.36)*	*(0.01)*	*(0.42)*	*(0.13)*	*(0.03)*
	PL-GRAN	52.2 a	7.1	8.48	75.9 a	11.6	12.41 b	0.07 a	2.88 a	1.37	0.49
		(4.7)	*(0.6)*	*(1.33)*	*(2.9)*	*(0.3)*	*(2.59)*	*(0.00)*	*(0.64)*	*(0.11)*	*(0.22)*
Meso	PL-CONT	9.4	1.1	6.91	17.7	2.8	12.95	0.01	0.34	1.46	0.26
		(1.7)	*(0.6)*	*(1.99)*	*(1.4)*	*(0.6)*	*(2.40)*	*(0.01)*	*(0.16)*	*(0.61)*	*(0.14)*
	PL-FERT	13.4	1.3	8.13	17.3	2.7	10.40	0.01	0.69	1.01	0.43
		(3.2)	*(0.3)*	*(3.49)*	*(1.9)*	*(0.7)*	*(3.17)*	*(0.01)*	*(0.40)*	*(0.31)*	*(0.32)*
	PL-GRAN	7.7	1.0	8.35	11.4	1.7	12.41	0.01	0.52	1.36	0.54
		(3.1)	*(0.4)*	*(0.83)*	*(4.4)*	*(0.9)*	*(1.10)*	*(0.00)*	*(0.37)*	*(0.30)*	*(0.25)*
Micro	PL-CONT	7.8	1.8	5.02	18.9	2.7	12.52	0.01	0.37 b	1.72	0.26
		(3.6)	*(0.4)*	*(0.02)*	*(6.5)*	*(1.3)*	*(1.59)*	*(0)*	*(0.03)*	*(0.50)*	*(0.10)*
	PL-FERT	6.5	0.7	7.37	10.9	1.9	12.36	0.01	0.32 b	1.37	0.49
		(2.4)	*(0.2)*	*(1.86)*	*(4.0)*	*(0.8)*	*(3.02)*	*(0)*	*(0.24)*	*(0.20)*	*(0.15)*
	PL-GRAN	8.4	1.1	5.52	18.6	2.7	12.20	0.01	0.61 a	1.54	0.40
		(1.5)	*(0.4)*	*(0.51)*	*(2.5)*	*(0.7)*	*(0.56)*	*(0)*	*(0.00)*	*(0.11)*	*(0.00)*

Table 12. Main biological characteristics of aggregate fractions. The values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. The numbers in parentheses and italic are standard deviation values.

6.3 Extracellular enzymatic activities

The macroaggregates of walnut fertirrigate and granular had higher extracellular enzymatic activities than those of walnut control site, with the exception of β-xylosidase, arylsulfatase and phosphodiesterase which were not affect by soil management (Table 13). This trend was not observed in the meso- and microaggregates, where enzymatic activities related to C N, P and S cycle were similar among sites.

		Extracellular enzymatic activity nmol MUF h^{-1} g^{-1}_{soil}							
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
Macro	PL-CONT	52.1 b	2.9 b	16.1 b	8.9	5.0 b	44.8	78.7 b	22.3
		(6.7)	*(0.7)*	*(7.9)*	*(3.6)*	*(1.9)*	*(9.6)*	*(15.6)*	*(7.0)*
	PL-FERT	68.1 b	5.8 a	52.5 a	16.3	14.1 ab	45.4	129.6 ab	35.1
		(2.9)	*(0.8)*	*(0.8)*	*(5.0)*	*(0.9)*	*(4.2)*	*(14.4)*	*(5.2)*
	PL-GRAN	104.0 a	4.3 ab	38.2 ab	18.7	18.1 a	72.3	146.7 a	42.3
		(2.7)	*(0.2)*	*(9.5)*	*(0.2)*	*(4.2)*	*(6.3)*	*(8.7)*	*(6.0)*
Meso	PL-CONT	14.9	1.0	7.5	3.2	1.7	16.5	27.6	8.2
		(2.4)	*(0.2)*	*(1.8)*	*(0.9)*	*(0.7)*	*(1.2)*	*(1.5)*	*(1.8)*
	PL-FERT	27.8	1.7	10.6	6.4	3.9	15.6	34.7	12.2
		(5.4)	*(0.5)*	*(2.1)*	*(0.3)*	*(1.8)*	*(3.6)*	*(9.6)*	*(1.5)*
	PL-GRAN	12.1	0.9	6.5	4.0	2.7	10.7	23.4	6.6
		(4.8)	*(0.6)*	*(3.3)*	*(1.6)*	*(1.6)*	*(3.4)*	*(5.5)*	*(2.6)*
Micro	PL-CONT	20.3	1.3	8.7	4.2	2.0	21.8	35.6	11.2
		(6.8)	*(0.2)*	*(0.6)*	*(0.5)*	*(0.1)*	*(7.2)*	*(8.3)*	*(1.6)*
	PL-FERT	14.4	1.0	7.4	3.2	2.4	10.0	25.6	6.6
		(6.9)	*(0.6)*	*(3.7)*	*(2.1)*	*(1.0)*	*(6.9)*	*(7.0)*	*(3.7)*
	PL-GRAN	31.4	1.7	11.6	4.6	4.4	21.4	37.6	11.5
		(1.1)	*(0.5)*	*(1.2)*	*(1.3)*	*(1.0)*	*(0.1)*	*(3.7)*	*(0.1)*

Table 13. Enzymatic activities of the aggregates fraction β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE). The values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. The numbers in bracket and italics are standard deviation values.

Tables 14 and 15 shows the enzymatic activities expressed per unit of C_{mic} and C_{org}, thus the enzymatic activities expressed on the basis of different unit of C pools (i.e., microbial and total, respectively). The values related to the labile C (Table 14) did not confirm higher enzyme activity of C, N, P and S cycle in macroaggregates of the urea-added sites. In fact the different management

did not influence any enzymatic activity expressed for unit to C_{mic}. Conversely, the mesoaggregates showed differences among sites for some activities related to the C and S cycle. Walnut fertirrigate had in fact higher value of β-glucosidase (2.09 nmol MUF $g^{-1} h^{-1}$) than walnut control and granular (1.59 and 1.57 nmol MUF $g^{-1} h^{-1}$, respectively) but lower value of arylsulfatase activity (for walnut fertirrigate was 1.17 nmol MUF $g^{-1} h^{-1}$, for walnut control and granular was 1.77 and 1.42 nmol MUF $g^{-1} h^{-1}$, respectively). These differences were not observed in the microaggregates, where the values of all specific activities were similar among sites. If the specific enzymatic activities were expressed on the basis of organic C (Table 15), the values of the enzymatic activities were not different among soil management both in macro- and mesoaggregates. In microaggregates, the values of β-glucosidase expressed per unit of C_{org} were different among sites, with walnut granular and fertirrigate sites that had higher values (20.7 and 15.7 nmol MUF $g^{-1} h^{-1}$, respectively) than walnut control (13.5 nmol MUF $g^{-1} h^{-1}$).

		Extracellular enzymatic activity nmol MUF h^{-1} g^{-1}/ C_{mic} kg^{-1}							
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SUF	PME	PDE
Macro	PL-CONT	1.75	0.10	0.54	0.20	0.17	1.51	2.65	0.75
		(0.28)	*(0.02)*	*(0.28)*	*(0.08)*	*(0.07)*	*(0.37)*	*(0.60)*	*(0.26)*
	PL-FERT	1.35	0.12	1.04	0.32	0.28	0.90	2.57	0.70
		(0.02)	*(0.02)*	*(0.06)*	*(0.11)*	*(0.03)*	*(0.12)*	*(0.39)*	*(0.13)*
	PL-GRAN	2.00	0.08	0.74	0.36	0.35	1.39	2.82	0.82
		(0.13)	*(0.00)*	*(0.25)*	*(0.04)*	*(0.11)*	*(0.01)*	*(0.09)*	*(0.19)*
Meso	PL-CONT	1.59 b	0.10	0.80	0.34	0.18	1.77 a	2.97	0.86
		(0.04)	*(0.00)*	*(0.05)*	*(0.04)*	*(0.04)*	*(0.20)*	*(0.39)*	*(0.04)*
	PL-FERT	2.09 a	0.13	0.80	0.50	0.28	1.17 b	2.59	0.93
		(0.10)	*(0.01)*	*(0.03)*	*(0.14)*	*(0.07)*	*(0.01)*	*(0.10)*	*(0.11)*
	PL-GRAN	1.57 b	0.12	0.82	0.51	0.34	1.42 ab	3.00	0.86
		(0.01)	*(0.03)*	*(0.10)*	*(0.01)*	*(0.07)*	*(0.12)*	*(0.18)*	*(0.01)*
Micro	PL-CONT	2.69	0.18	1.23	0.58	0.28	2.89	4.83	1.55
		(0.37)	*(0.05)*	*(0.49)*	*(0.21)*	*(0.12)*	*(0.41)*	*(1.16)*	*(0.51)*
	PL-FERT	14.4	1.0	7.4	3.2	2.4	10.0	25.6	6.6
		(6.90)	*(0.61)*	*(3.72)*	*(1.10)*	*(1.04)*	*(3.96)*	*(6.01)*	*(3.70)*
	PL-GRAN	31.4	1.7	11.6	4.6	4.4	21.4	37.6	11.5
		(1.11)	*(0.50)*	*(1.22)*	*(1.13)*	*(1.03)*	*(0.19)*	*(3.17)*	*(0.19)*

Table 14. Specific activities of β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE) expressed per unit of C_{mic}. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. The numbers in bracket and italics are standard deviation values.

		Extracellular enzymatic activity nmol MUF h^{-1} g^{-1}/ C_{org} kg^{-1}							
Aggregate size fraction	ID	β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
Macro	PL-CONT	13.71	0.76	4.36	2.38	1.33	11.87	20.84	5.94
		(3.88)	*(0.29)*	*(2.73)*	*(1.31)*	*(0.70)*	*(4.32)*	*(7.28)*	*(2.73)*
	PL-FERT	12.44	1.07	9.62	3.05	2.58	8.37	23.92	6.49
		(1.05)	*(0.28)*	*(1.36)*	*(1.30)*	*(0.49)*	*(1.81)*	*(5.63)*	*(1.77)*
	PL-GRAN	17.04	0.69	6.47	3.08	3.06	11.76	23.94	7.08
		(3.76)	*(0.14)*	*(3.11)*	*(0.79)*	*(1.42)*	*(1.88)*	*(4.49)*	*(2.70)*
Meso	PL-CONT	10.94	0.71	5.56	2.37	1.29	12.03	20.15	6.01
		(2.86)	*(0.22)*	*(1.90)*	*(0.93)*	*(0.63)*	*(2.14)*	*(3.21)*	*(1.97)*
	PL-FERT	16.85	1.06	6.43	3.80	2.42	9.51	21.23	7.37
		(6.52)	*(0.51)*	*(2.52)*	*(0.62)*	*(1.55)*	*(3.98)*	*(9.86)*	*(2.33)*
	PL-GRAN	13.09	0.98	6.87	4.28	2.85	11.77	25.11	7.15
		(1.35)	*(0.31)*	*(1.53)*	*(0.49)*	*(0.84)*	*(0.20)*	*(3.97)*	*(0.63)*
Micro	PL-CONT	13.50b	0.91	6.17	2.92	1.42	14.52	24.26	7.77
		(1.89)	*(0.27)*	*(2.50)*	*(1.05)*	*(0.59)*	*(2.08)*	*(5.90)*	*(2.58)*
	PL-FERT	15.67ab	1.01	8.04	3.30	2.63	10.17	27.95	6.92
		(1.92)	*(0.04)*	*(0.82)*	*(0.23)*	*(0.45)*	*(1.25)*	*(3.79)*	*(0.14)*
	PL-GRAN	20.71a	1.09	7.63	3.01	2.88	14.13	24.72	7.57
		(1.13)	*(0.20)*	*(0.08)*	*(0.59)*	*(0.39)*	*(1.18)*	*(0.22)*	*(0.61)*

Table 15. Specific activities of β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE) expressed per unit C_{org}. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. The numbers in bracket and italics are standard deviation values.

6.4 Total DNA and fungi diversity

In all examined sites, the largest amount of total extracted DNA were in meso- and microaggregates (table 16), which ranged from 0.55 to 1.43 μg g^{-1}soil in the mesoaggregates, and from 0.64 to 0.95 μg g^{-1}soil in the microaggregates. The different management did not influence the total extracted DNA in macro- and microaggregate, but in the mesoaggregates it was found that walnut granular had higher values of DNA than walnut fertirrigate and control (average values of extracted DNA: 1.88, 0.60 and 0.57 μg g^{-1}soil, respectively). The Shannon-Wiener (H') and Eveness index (J') were similar among sites in all aggregate fractions, as well the values of the extracted DNA/C_{mic} ratio.

Aggregate size fraction	ID	Sampling	DNA_{tot} μg g^{-1}_{soil}	DNA_{tot}/ C_{mic} μg g^{-1}_{Cmic}	*H'*	*J'*
Macro	PL-CONT	1	0.40 *(0.25)*	0.01	0.72	0.83
		2	0.66 *(0.48)*	0.02	0.95	0.90
	PL-FERT	1	0.80 *(0.34)*	0.02	0.91	0.90
		2	0.89 *(0.12)*	0.02	0.99	0.80
	PL-GRAN	1	1.18 *(0.10)*	0.02	1.05	0.83
		2	0.82 *(0.24)*	0.01	0.83	0.77
Meso	PL-CONT	1	0.59 *(0.19)* b	0.07	0.34	0.70
		2	0.55 *(0.13)* b	0.05	1.09	0.91
	PL-FERT	1	0.66 *(0.23)* b	0.04	1.02	0.95
		2	0.55 *(0.16)* b	0.05	1.06	0.96
	PL-GRAN	1	1.43 *(0.13)* a	0.15	1.04	0.89
		2	1.17 *(0.20)* a	0.21	0.73	0.87
Micro	PL-CONT	1	0.86 *(0.77)*	0.08	0.87	0.89
		2	0.81 *(0.27)*	0.15	1.06	0.93
	PL-FERT	1	0.95 *(0.23)*	0.20	0.87	0.94
		2	0.97 *(0.50)*	0.12	0.89	0.89
	PL-GRAN	1	0.64 *(0.24)*	0.07	0.89	0.91
		2	0.59 *(0.12)*	0.08	0.80	0.79

Table 16. Amounts of DNA extraction efficiency (DNA_{tot}), total DNA/C_{mic} ratio, Shannon Wiener (H') and Eveness (J') index in aggregate fractions. The values were expressed in proportion of the weight of the single aggregates fraction on the whole soil mass. The letters show the significant differences at *p* level < 0.05 Tukey test among walnut control, fertirrigate and granular sites in macro, meso and microaggregates. The numbers in parentheses and italics are standard deviation values.

The effect of the different urea fertilization management on the clustering analysis of the DGGE patterns of all aggregate fractions was reported in Fig 7a-c. Cluster analysis of macroaggregates (Fig 7a) indicated a first division between macroaggregates of walnut control and walnut both

fertirrigate and granular (similarity <40%) with exception of one walnut control sample. A second clustering level separated the two field replications within the walnut control site (similarity <50%). A further clustering level identified two groups (similarity <50%) where no distinct separation was reported among the field replications of walnut fertirrigate and granular site.

In the cluster analysis of mesoaggregates (Fig 7b) there was a first clustering, where all field replications of walnut control (PL-CONT1 and PL-CONT2) and the second sampling of the walnut granular (PL-GRAN2) were separated from the field replications of walnut fertirrigate (PL-FERT 1and PL-FERT2) and the first sampling of the walnut granular (PL-GRAN1), with a <30% similarity. A second clustering level separated the walnut control site from the walnut granular (second sampling PL-GRAN2), with a <30 % similarity. The third clustering level separated the walnut fertirrigate site from the walnut granular (first sampling PL-GRAN1), with a < 40% similarity.

The same pattern was observed for the microaggregates cluster analysis (Fig 7c) that in the first level separated the field replications of walnut control (PL-CONT1 and PL-CONT2) and the second sampling of the walnut fertirrigate (PL-FERT2) from the field replications of walnut granular (PL-GRAN 1and PL-GRAN2) and the first sampling of the walnut fertirrigate (PL-FERT 1), with a similarity <30 %.

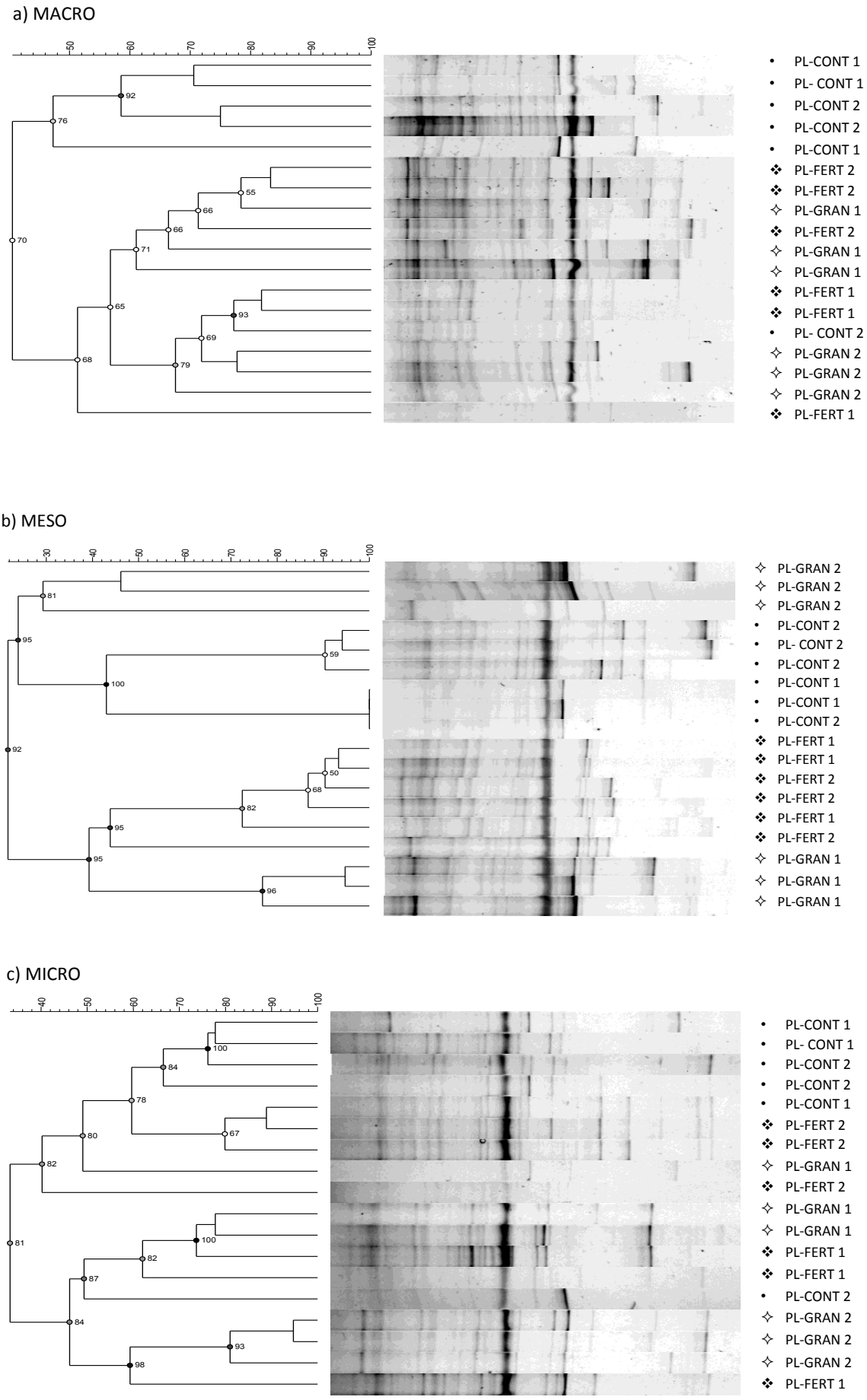


Figure 7. Effect of the different management on the clustering analysis of the DGGE patterns of

macroaggregates (7.a) mesoaggregates (7.b) and microaggregates (7.c). PL-CONT, PL-FERT and PL-GRAN indicate walnut control, fertirrigate and granular sites and the number 1 and 2 indicate the two field replications on each sites (1° sapling and 2°sampling).

6.5 Physical parameters

The physical parameters of aggregates are shown in Table 17. The data of the particle size distribution showed that the macroaggregates of walnut fertirrigate and granular were clay and sand-enricher than those of walnut control site (clay: 16.6, 16.8 and 13.6 g kg^{-1} for the walnut fertirrigate, granular and control, respectively; sand: 27.6, 26.7 and 23.7 g kg^{-1}, respectively). The mesoaggregates from walnut control and fertirrigates sites had higher values of clay and coarse sand than those from walnut granular (clay: 4.3, 4.9 and 2.6 g kg^{-1} for the walnut control, fertirrigate and granular, respectively; coarse sand: 0.8, 0.9 and 0.5 g kg^{-1}, respectively). In microaggregates, the walnut fertirrigate was instead clay poorer with respect to walnut control and granular (clay: 4.9, 2.6 and 4.4 g kg^{-1} for the walnut control, fertirrigate and granular, respectively).

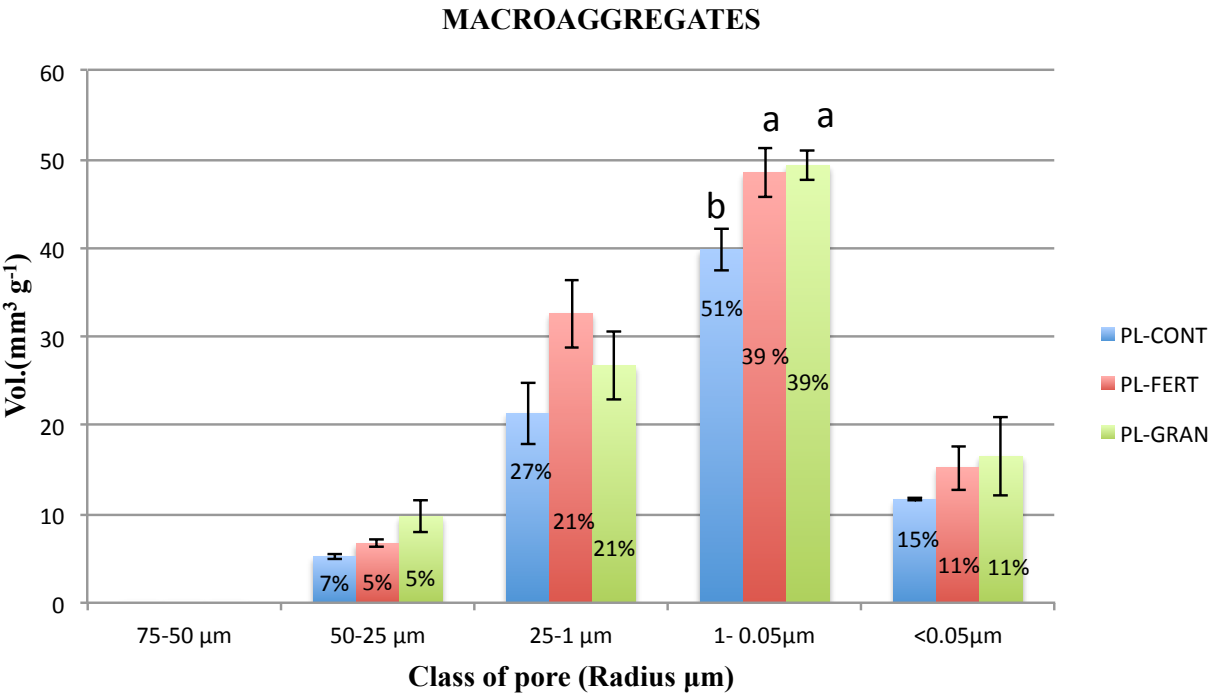
Significant differences were found also in the total pore volume (V_{Hgtot}) and pore size distribution. In macroaggregates, the values of V_{Hgtot} were higher in walnut fertirrigate and granular than in control (103.08 and 102.35 *vs*. 77.96 mm^3 g^{-1} in the walnut control; Table 17). In mesoaggregates, the values of $V_{Hg\ tot}$ were higher in walnut control and fertirrigate (40.19 and 45.12 *vs.* 21.88 mm^3 g^{-1} in walnut granular). While in microaggregates the values of V_{Hgtot} were higher in walnut control and granular (27.93 and 23.80 *vs.* 17.83 mm^3 g^{-1} in the walnut fertirrigate). The values of the specific surface area of the pore (SSA_{tot}) were always similar among the sites.

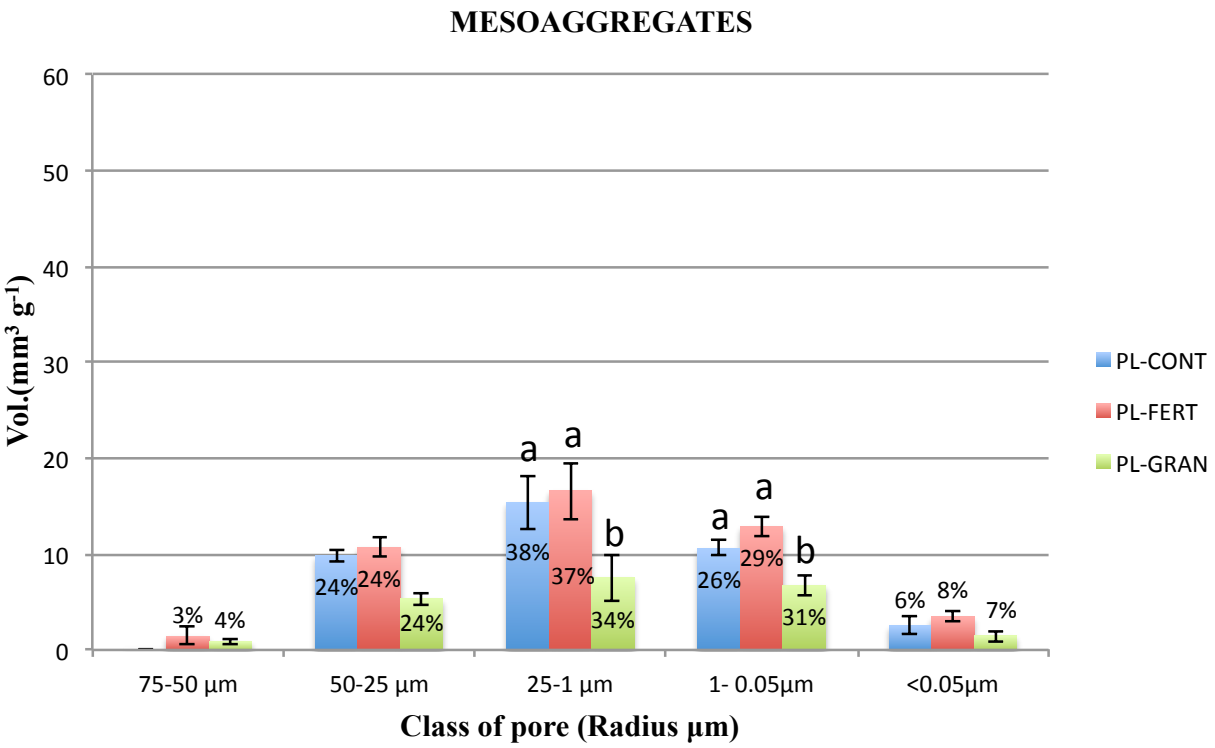
As regards to the pore size distribution, in all the aggregate classes the pores were unimodally distributed (Fig 8). Independently from urea fertilization management, small pores of 1-0.05 μm and large pore of 25-1 μm in size were the most frequent in macroaggregates, and in meso- and microaggregates, respectively. In the macroaggregates, the pore size distribution was similar among soil management, with the exception of higher presence of 1-0.05 pore in walnut fertirrigate and granular that walnut control, thus of the most represented pore class. The higher porosity in walnut fertirrigate and granular macroaggregates appeared therefore ascribable to small pores. In mesoaggregates, the highest values of $V_{Hg\ tot}$ observed in walnut control and fertirrigate were due both to large 25-1 μm and small pores <1 μm, as visible in Fig 8. The microaggregates were characterized by the lack of 75-50 and 50-25 μm pore classes. However, the other classes of pore, especially 25-1 and 1-0.05 μm pore classes, showed significant differences among sites, and walnut

control and granular had higher values of both classes. Therefore, as in mesoaggregates, higher porosity was due both to large and small pores.

Aggregate size fraction	ID	Clay (<2 µm) g kg^{-1}_{soil}	Total sand (2-0.05 mm) g kg^{-1}_{soil}	Coarse Sand (2-0.2mm) g kg^{-1}_{soil}	$V_{Hg\ tot}$ $mm^3 g^{-1}_{soil}$	SSA_{tot} $m^2 g^{-1}_{soil}$
Macro	PL-CONT	13.6 b	23.7	2.2	77.96 b	2.41
		(5.0)	*(1.3)*	*(0.7)*	*(3.01)*	*(0.23)*
	PL-FERT	16.6 a	27.6	2.5	103.08 a	3.13
		(1.3)	*(0.3)*	*(0.5)*	*(4.23)*	*(0.21)*
	PL-GRAN	16.8 a	26.7	2.4	102.35 a	2.62
		(7.1)	*(2.0)*	*(1.3)*	*(1.00)*	*(0.71)*
Meso	PL-CONT	4.3 a	7.0	0.8 a	40.19 a	0.39
		(0.8)	*(0. 5)*	*(0.3)*	*(9.12)*	*(0.14)*
	PL-FERT	4.9 a	6.7	0.9 a	45.12 a	0.85
		(0.7)	*(0.5)*	*(0.1)*	*(13.01)*	*(0.20)*
	PL-GRAN	2.6 b	4.0	0.5 b	21.88 b	0.29
		(0.3)	*(2.3)*	*(0.3)*	*(9.23)*	*(0.10)*
Micro	PL-CONT	4.9 a	9.0	0.2	27.93 a	0.14
		(0.7)	*(3.6.)*	*(0.1)*	*(9.45)*	*(0.11)*
	PL-FERT	2.6 b	4.4	0.1	17.83 b	0.45
		(0.1)	*(1.9)*	*(0.1)*	*(6.25)*	*(0.21)*
	PL-GRAN	4.4 a	7.2	0.3	23.80 a	0.37
		(0.7)	*(1.7)*	*(0.1)*	*(1.12)*	*(0.16)*

Table 17. Main physical characteristics of the aggregates. Total sand: coarse+fine sand; V_{Hgtot}: total volume of pore; SSA_{tot}: specific surface area of the pore. The values are expressed as weighted average, and thus as proportion of the whole soil mass taking into account the mass of aggregate fraction. The letters show the significant differences at *p* level <0.05 Tukey test among walnut control, fertirrigate and granular sites in macro-, meso- and microaggregates. Numbers in parentheses and italics are standard deviation values.


MACROAGGREGATES
Vol.(mm³ g⁻¹)
60
50
40
30
20
10
0
a
a
b
51%
39 %
39%
27%
21%
21%
7%
5%
5%
15%
11%
11%
75-50 µm
50-25 µm
25-1 µm
1- 0.05µm
<0.05µm
Class of pore (Radius µm)
PL-CONT
PL-FERT
PL-GRAN


MESOAGGREGATES
Vol.(mm³ g⁻¹)
60
50
40
30
20
10
0
3%
4%
24%
24%
24%
a
a
b
38%
37%
34%
a
a
b
26%
29%
31%
6%
8%
7%
75-50 µm
50-25 µm
25-1 µm
1- 0.05µm
<0.05µm
Class of pore (Radius µm)
PL-CONT
PL-FERT
PL-GRAN

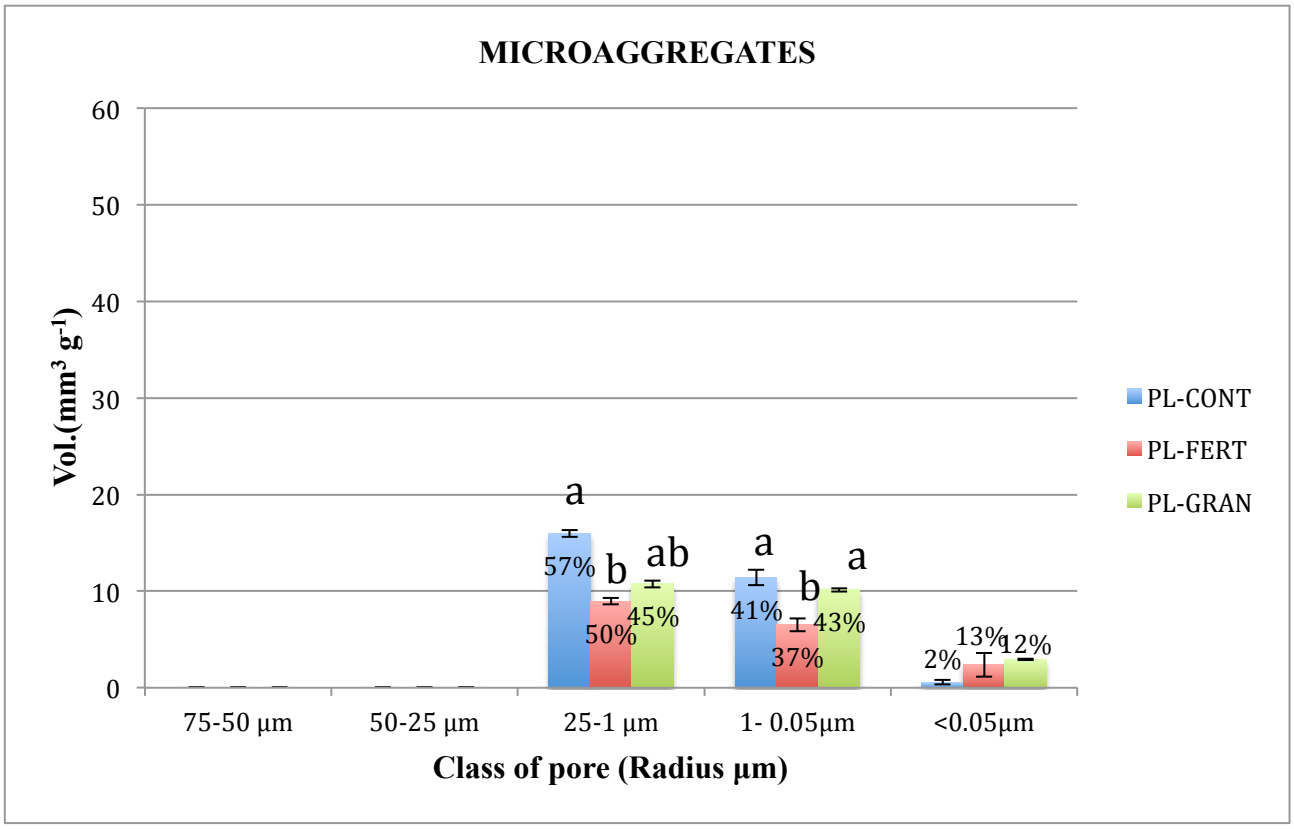


Figure 8. Pore size distribution expressed as volume (mm^3 g^{-1}soil). The numbers inside the graph show the pore size distribution expressed as percentage [(volume of pore class/V_{HgTot})·100]. The letters show the significant differences at p level <0.05 Tukey test among walnut sites in macro-, meso- and microaggregates

6.6 Discussion

Nitrogen fertilization effects on macroaggregates

Macroaggregates represented more that 67% of total soil mass in our sites. The same aggregate size distribution between sites demonstrated that soil aggregate formation was not influenced by urea addition. This was in contrast with the expected N addition effect on aggregate size distribution. In fact it is well known that fertilizer application improves crop yields, increases organic matter returns and raises soil organic matter levels as compared with unfertilized crops (Brar et al., 2015). For these reasons, N fertilization was expected to positively influence soil aggregate genesis (Haynes et al., 1998). Moreover, again contrary to what was expected, in aggregates fertilization (Walnut fertirrigate and granular sites) did not increase the amount of C_{org}. We thus hypothesized that the lack of any direct nitrogen input effect on the organic matter content was the reason why we failed to observe an effect on aggregate distribution.

The lack of any N addition effect on the total organic carbon content was probably due to the quantity of urea supplied (perhaps the amount of urea was too low to observe any positive effect).

However, even though no effect was visible on the total C pool, a greater root development was observed, enhanced by the nitrogen supply (data not shown). This could lead to a higher amount of root exudates affecting the C labile pool (C_{mic} and C_{extr}). And indeed, in the walnut fertirrigate and granular sites we found higher values of C_{mic} and C_{extr} as well as lower degree of organic matter transformation (as recorded by lower $\delta^{13}C$ values) than in the walnut control site. Thus, however low the amount of urea, it was enough to affect both the C labile pool and the degree of soil organic transformation.

The increase of microbial biomass (C_{mic}) in the walnut fertirrigate and granular sites did not correspond to any increase in the microbial quotient (C_{mic}/C_{org} ratio). In point of fact C_{mic}/C_{org} ratio values did not differ among sites. Anderson and Domsch (1989) reported that the microbial quotient reflects the C substrate availability for soil microorganisms. Since, the supply of urea was not such as to affect the substrate availability for soil microorganisms, the establishment of energetic metabolism among microorganisms was favoured (Sparling, 1992). This was also confirmed by qCO_2 which indicated a similar level of microbial biomass specific activity among sites.

Application of mineral N can directly affect the microbial production of soil enzymes; and this possible effect varies with the type of soil and enzyme as well as with the kind of enzymatic reaction (Iyyemperumal and Shi., 2008). On the other hand, N fertilization, especially in mineral forms, may have an indirect effect on the activities of soil enzymes via greater root development (Lee et al., 2003). In macroaggregates, we did observed that fertilization seemed to stimulate biological activity, in particular the enzymatic activities involved the C and N cycles (β-glucosidase, α-glucosidase, β-cellobiosidase, N-acetyl β-glucosaminidase), though also the P cycle (Phosphomonoesterase). Indeed, in macroaggregates from the walnut fertirrigate and granular sites we found higher enzymatic activities than in the walnut control site. Some studies have shown that N fertilization can accelerate the activity of some C, N and P cycle enzymes, like cellulase and phosphatase (Sinsabaugh et al., 2005). Turner et al. (2002) reported that the increased phosphatase activity in response to N addition probably reflects increased P demand, a likely consequence of reduced N limitation on microbial activity. By contrast, unlike the results of Siwik-Ziomek et al. (2013) and Iyyemperumal and Shi (2008), who found that addition of more than 100 kg N ha^{-1} coincided with boosted activity of arylsulfatase and acid phosphatase, in our study there were no differences among treatments in terms of arylsulfatase and phosphodiesterase activities. However, because the nitrogen supply in both walnut fertirrigate and granular sites was 90 kg N ha^{-1}, this would be further evidence of the low dose of urea supplied.

The specific enzyme activities, expressed per unit both of C_{org} and of C_{mic}, were similar among the walnut sites. These results confirmed that the nitrogen supply was not enough to modify the soil organic matter or the metabolic status of the microbial community, thus integrating the information obtained from using qCO_2 (Landi et al., 2000). This was further strengthened by the same amount of DNA being extracted from macroaggregates of the different sites, by the 40% similarity between walnut fertilized and walnut control, and by the same values of the diversity index (Shannon-wiener index H').

As regards physical properties, our walnut fertirrigate and granular sites showed higher values of total porosity volume (V_{Hgtot}) and of the small pore class (1-0.05 μm). This could be due to the higher amount of clay particles. In fact, according to Attou and Le Bissonnais (1998), the small pore class corresponds to pore originating from clay-clay interactions and related therefore to the amount of clay particles. As discussed in chapter 5, higher fine porosity could lead to greater physical protection of organic matter, a further reason limiting microbial activity and, consequently, degradation of organic matter in the walnut fertirrigate and granular sites.

Nitrogen fertilization effects on meso- and microaggregates

In meso- and microaggregates we did not observe any positive effect ascribable to urea addition. Unlike macroaggregates, no differences were observed among sites as to the degree of soil organic transformation and the amount of the C labile pool.

All enzymatic activities were similar in walnut sites, with the exception of β-glucosidase and arylsulfatase activities expressed per unit C_{mic} in mesoaggregates and β-glucosidase expressed per unit C_{org} in microaggregates. This, however, appeared more related to textural differences rather than differences in N fertilization management. For, as is known, enzyme activities are unequally associated with different particle size fractions (e.g., Qin et al., 2010, Saviozzi et al., 2007). In general, the enzymes related to C compound transformation, such as β-glucosidase (Marx et al., 2005), predominate in coarse sand size classes, while the enzymes involved in the S cycle (arylsulfatase) relate to the clay fraction (Stemmer et al., 1999a). In N fertilized mesoaggregates, the specific β-glucosidase activity (higher in walnut fertirrigated and lower in walnut granular mesoaggregates) may have been related to the presence of coarse sand. The different distribution of the specific β-glucosidase activity could therefore be attributed, at least in the case of N-fertilized mesoaggregates, to the different particle size distribution and probably to differently mineralized particulate organic matter in the coarser particle size fraction, as suggested by Stemmer et al.

(1998). In the walnut control mesoaggregates, lower specific β-glucosidase activity corresponded instead to the higher amount of coarse sand. However, in the control site higher specific arylsulfatase activity seemed related to higher clay content (Qin et al., 2010). In microaggregates no relationships were observed between specific enzyme activities and particle size distribution, and no speculation between them can be attempted. The effect of particle size distribution on specific enzyme activities, if any in our samples, seems not so relevant to any activities. However, we cannot rule it out , at least in mesoaggregates.

Particle size distribution, however, affected the pore size distribution, both in meso- and microaggregates. In fact, the most heavily represented pore class (25-1 μm in size) was affected by clay and/or coarse sand content. This was in agreement with Attou and Le Bissonnais (1998), who state that this class of pores originates from clay-sand interaction. The values of extracted DNA in meso- and microaggregates was very high; the walnut control and fertirrigate sites showed lower values of extracted DNA than walnut granular in mesoaggregates, but this could correlate with the higher amount of clay, because DNA gets adsorbed and bound on clay minerals and other particles, and appears to be resistant to degradation (Alvarez et al., 1998)

6.7 Conclusions

From biophysical properties investigated, it was evident that, in all aggregate fractions, no effect was produced by urea addition on the of the total organic matter content, probably due to the low quantity of fertilizer used. Urea addition did have direct (on enzyme activity of C- cycle) and indirect effects (i.e. through root development) on the quality of the organic matter. In macroaggregates, these two effects may contribute to the enhancement of enzymatic activities and increase in the labile C pool. The degree of degradation of organic matter, evaluated by the $\delta^{13}C$, appears more related to physical occlusion due to a higher presence of clay particles and, consequently, higher fine porosity.

The effect of the urea supply was not observed in smaller aggregates, however. Only in the mesoaggregates from walnut granular and fertirrigates sites was it possible to observe an increase in the specific enzymatic activity of β-glucosidase, but this was related to particle distribution rather than N addition. Mesoaggregates (meaning aggregates between 1 mm and 250 μm) behaved more like microaggregates, otherwise they were more influenced by the aggregative process than by management.

7. EVIDENCES OF RELATIONSHIPS BETWEEN INTRA-AGGREGATES STRUCTURE AND ORGANIC MATTER FEATURES BY OPTICAL MICROSCOPY AND SEM-EDS ANALYSIS

7.1 Micromorphology observation, image analysis of pore and organic components on macro- and mesoaggregate thin sections

The diameter of macroaggregates varies from 2.20 to 3.09 mm, while the mesoaggregates ranged from 0.55 to 0.65 mm (Table 18). Among sites, oak wood had larger aggregate size. In all sites, total porosity was <10% (Table 18), therefore at the lowest limit for good soil structural condition (Pagliai et al, 1988). In general, macroaggregates had higher porosity than mesoaggregates, which ranged from 5.96 to 9.08% and from 3.70 to 6.71%, respectively. Among sites, aggregates from alfalfa and walnut granular sites had the highest porosity, while walnut control had the lowest one. These features suggested that macroaggregates from alfalfa and walnut granular had better structural condition than the other samples, being also the closest to the limit of total porosity (9.08 and 8.19%, respectively) defined as good physical conditions by Pagliai et al. (1988).

Among sites, on the average oak wood and alfalfa had higher percentage of organic forms than walnut sites (12.71 and 12.13% in oak and alfalfa *vs.* 9.29, 7.04 and 9.56% in the walnut sites; Table 18). The percentage of organic forms however varied from 4.19 to 8.27 and from 7.90 to 17.75 in macro- and mesoaggregates, respectively. This data suggested that soil management was not the only significant factor affecting organic matter content, as interaction between site and aggregate fraction was significant ($p<0.01$).

The perimeter of organic matter in contact with the pores was normalized for the surface of organic forms, as described in the Materials and Methods section. The obtained index, called exposure index (EI, mm^{-1}; Table 18), measures the degree of organic matter interaction with the pore system, and thus the organic matter occlusion in the aggregate matrix. The EI gives us, therefore, information about the potential availability of organic matter for microbial biomass, but does not provide information on quality of organic matter. Higher values of EI correspond to longer perimeter of organic matter in contact to pores with respect to the organic matter surface. This means higher potential availability of organic matter for microbial biomass. The values of EI (Table 18) showed significant differences among sites, where the alfalfa and oak wood showed higher index (0.62 and 0.68 mm^{-1} in oak and alfalfa *vs.* 0.27, 0.43 and 0.35 mm^{-1} for the walnut sites). Furthermore, it was possible to observe that mesoaggregates had higher EI values, and thus that

higher surface of organic matter was in contact with the pore system in mesoaggregates than in macroaggregates. Indeed the EI values ranged from 0.29 to 0.79 mm^{-1} in mesoaggregates, while it ranged from 0.25 to 0.59 mm^{-1} in macroaggregates.

In the macroaggregates, the pore size classes, classified into six categories (<25, 25-50, 50-75, 75-100, 100-200, 200-350 μm in equivalent pore radius) had a bimodal distribution in all sites (Fig 9a). Independently from soil management, the pores of <25 and 25-50 μm in size were the most frequent in macroaggregates, and the classes ranging from 100 to 350 μm represented the second peak of bimodal distribution. Also in mesoaggregates, the pore size distribution was bimodal (Fig 9b), with a first peak in pores <25 μm and a second peak in 75-100 μm. Walnut control and fertirrigate sites were however an exception. In these sites, pore larger than 50 μm were missing, and pore size distribution was unimodal. Among bimodality distributed mesoaggregate, oak wood and alfalfa had the most frequent pores in the 75- 100 μm size class, while walnut granular in the <25 μm size class. Furthermore, in all mesoaggregates the pores >100 μm were not found.

Site	Aggregate fraction	n	Aggregate diameter mm	Porosity %	Organic forms %	EI mm^{-1}
MO-W	MACRO	12	3.09	5.96	8.27	0.59
			0.90	*1.67*	*1.29*	*0.28*
MO-W	MESO	23	0.65	4.42	17.16	0.65
			0.09	*0.97*	4.48	*0.19*
mean				5.19 **bc**	12.71**a**	0.62 **ab**
MO-A	MACRO	12	2.20	9.08	6.51	0.56
			0.45	*1.00*	*0.95*	*0.20*
MO-A	MESO	29	0.55	6.71	17.75	0.79
			0.18	*1.43*	1.94	*0.15*
mean				7.90 **a**	12.13 **a**	0.68 **a**
PL-CONTR	MACRO	16	2.33	5.93	4.19	0.25
			1.02	*1.89*	*0.88*	*0.11*
PL-CONTR	MESO	38	0.57	3.70	14.39	0.29
			0.14	*0.78*	*2.99*	*0.26*
mean				4.82 **c**	9.29 **b**	0.27 **d**
PL-FERT	MACRO	12	3.04	6.81	6.17	0.34
			0.71	*0.85*	*1.28*	*0.13*
PL-FERT	MESO	37	0.55	5.33	7.90	0.52
			0.14	*1.09*	*1.07*	*0.27*
mean				6,07 **b**	7.04 **c**	0.43 **bc**
PL-GRAN	MACRO	9	2.86	8.19	5.58	0.34
			0.96	*1.43*	*1.10*	*0.22*
PL-GRAN	MESO	41	0.56	6.53	13.54	0.36
			0.17	*1.18*	*3.41*	*0.28*
mean				7.36 **a**	9.56 **b**	0.35 **cd**
Site			**	**	**	**
Aggregate fraction			**	**	**	**
Site X Aggregate fraction			**	ns	**	ns

Table 18. Diameter of aggregates, total porosity and organic matter percentage, and exposure index (EI) of the macro- and mesoaggregates in all sites. Numbers in italic show the standard deviation values. The letters show the significant difference and results of the two-away ANOVA calculated on the mean values (* $p<0.05$, ** $p<0.01$, ns not significant)

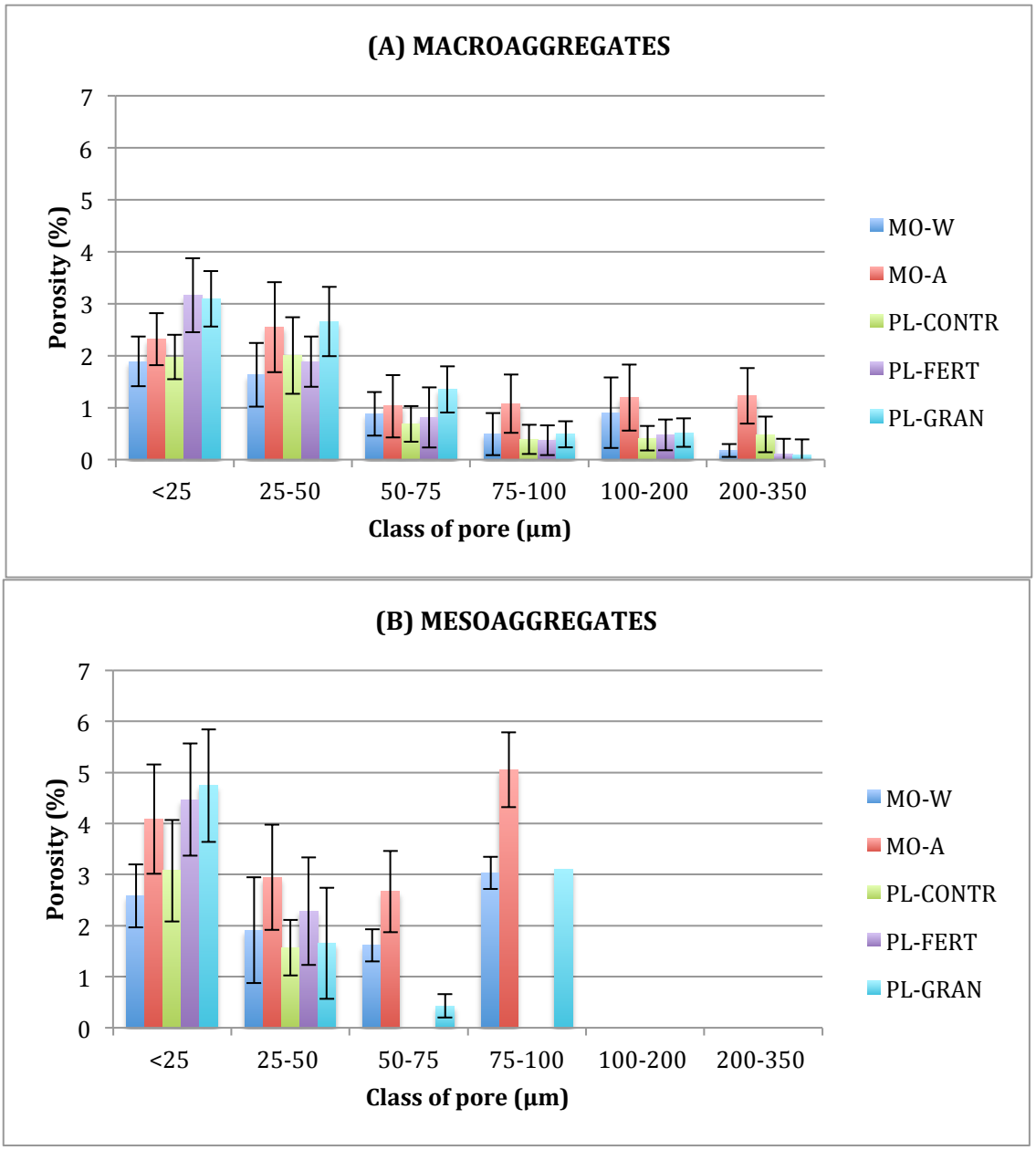


Figure 9. Pore size distribution in macro- (A) and mesoaggregates (B). The bars represent standard deviation values.

In Fig 10 we showed the relationships between organic forms amount and EI detected by image analysis of thin sections and some chemical and biochemical properties measured on aggregates (see chapter 5 and 6). The percentage of organic forms was positively related to the C organic content (r_s=0.673, $p < 0.05$; Fig 10a). We also found that the EI was related to the value of C/N ratio (r_s=0.675, $p<0.05$), $\delta^{13}C$ (r_s= - 0.821, $p<0.01$), and the average value of the enzyme activities related to the carbon cycle (r_s=0.888, $p<0.01$).

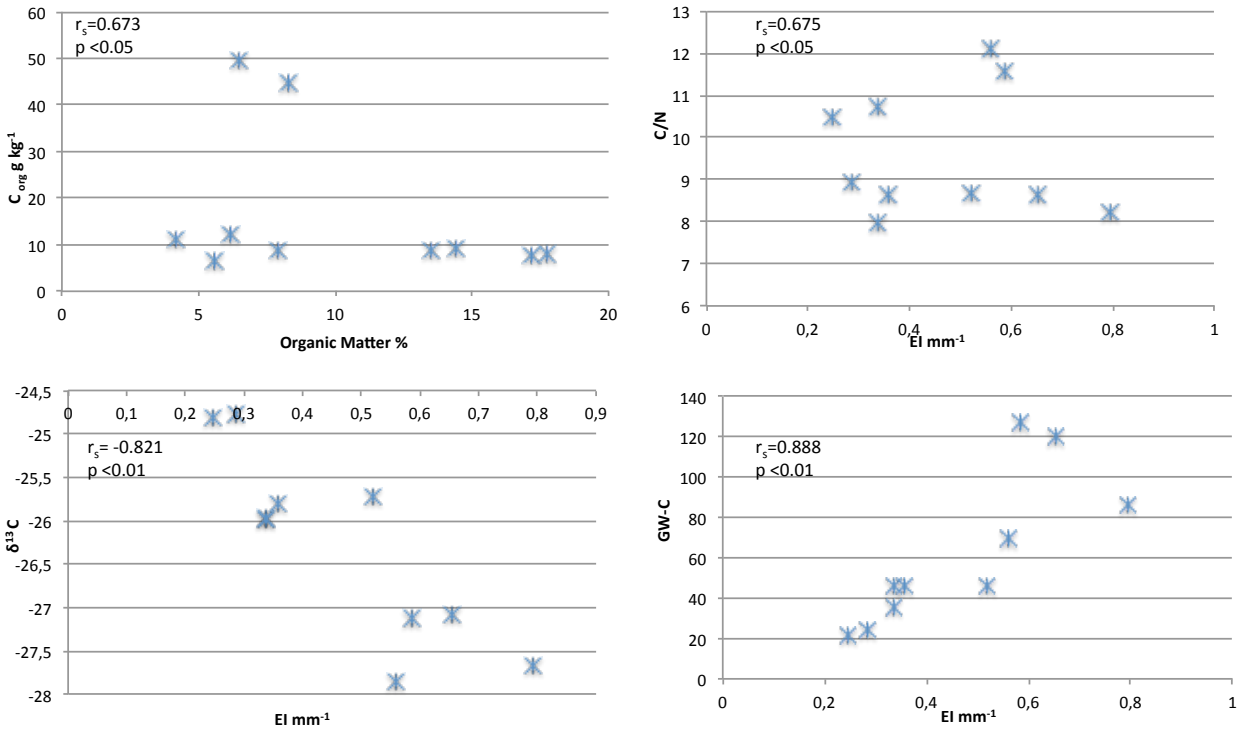


Figure 10. Relationship between intra-aggregates features measured by image analysis of aggregate thin section (i.e., organic matter and EI) and chemical and biochemical properties measured on aggregates (organic carbon, C/N ratio, $\delta^{13}C$ and average of the enzymatic activities related to the carbon cycle-GWC).

The distribution of the different organic forms, classified into moderately and strongly decomposed organs, and black, red and yellow amorphous organic matter, were shown in Table 19. In Fig 11 we reported an example of scanned image of a single area of interest and organic matter map with amorphous organic forms coloured according to their degree of decomposition.

Organ fragments (i.e., strongly and moderately decomposed organs) were detected in the macroaggregates of oak wood and alfalfa sites (Table 19). In the macroaggregates of oak wood site, the moderately decomposed organ fragments counted for 16.48% of the organic matter features, and represented the most abundant forms of organs. In the macroaggregates of alfalfa, it counted for a very low percentage (1.35%), and strongly decomposed organs (3.37%) represented the most abundant organs form. In all sites, organic matter was mainly present as amorphous organic matter forms, representing always more than 78% of organic compounds both in macro- and mesoaggregates. The amorphous forms were not however homogenous distributed among the forms (black, red and yellow). In all sites, we observed higher percentage of black and red amorphous organic material than that of yellow amorphous form. On the average, the higher values of black amorphous organic matter were observed in walnut granular and fertirrigate ($p<0.01$), and they corresponded to the 68.31 and 60.67% of organic forms, respectively. Among aggregate fractions,

the largest part of black amorphous forms was in mesoaggregates, where they ranged from 46.69 to 77.88%, while in macroaggregates they ranged from 26.85 to 58.74% ($p<0.01$). For the red amorphous forms, walnut control and alfalfa had higher amount than walnut granular (51.63, 45.97 and 27.56%, respectively; $p<0.05$), while oak and walnut fertirrigate had intermediate values (39.19 and 33.63%, respectively), with no differences between macro- and mesoaggregates. The yellow forms were not observed in all aggregates, and the site was the only significant factor ($p<0.05$) that affected their distribution. For this amorphous form, it was found that walnut control had the highest amount (14.83%), while alfalfa, walnut fertirrigate and granular had the lowest one (2.20, 11.41 and 8.26%, respectively) and oak had intermediate amount (11.55%).

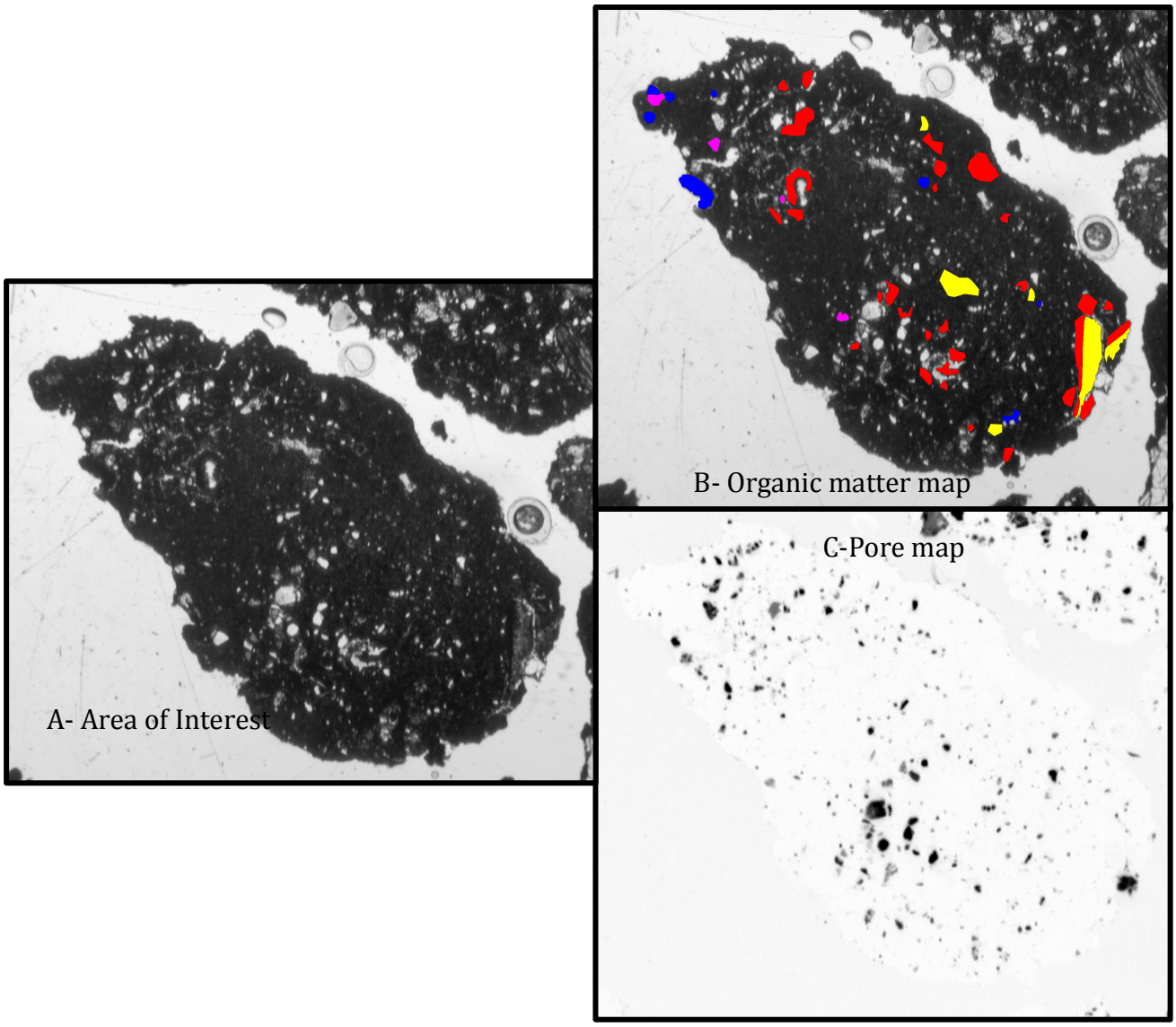


Figure 11. Example of scanned PPL- image for single area of interest. The A-image is the area of interest (single aggregate) of a thin section. In the B- image is the map of organic matter, where organic features are coloured according to their degree of decomposition (blue = black organic amorphous features, red and yellow are red and yellow organic amorphous features) and pink for moderately decomposed organs. The C-image shows the map of pores that is colour coded (white= soil matrix or organic matter, black= pores).

Site	Aggregate fraction	Σ organs %	Strongly decomposed organs %	Moderately decomposed organs %	Σ amorphous organic matter %	Black amorphous organic matter %	Red amorphous organic matter %	Yellow amorphous organic matter %
MO-W	MACRO	21.14	4.66	16.48	78.86	30.70	29.59	18.57
	dev	*16.07*	*6.67*	*9.39*	*41.91*	*19.23*	*12.68*	*10.00*
MO-W	MESO	absent	absent	absent	100.00	46.69	48.79	4.53
	dev	-	-	-	*56.63*	*28.97*	*24.80*	*2.87*
mean			4.66	16.48		38.69 **c**	39.19 **ab**	11.55 **ab**
MO-A	MACRO	4.71	3.37	1.35	95.29	32.45	62.84	absent
	dev	*10.50*	*7.20*	*3.30*	*31.95*	*16.88*	*15.07*	-
MO-A	MESO	absent	absent	absent	100.00	68.71	29.09	2.20
	dev	-	-	-	*50.30*	*27.58*	*16.80*	*5.92*
mean			3.37	1.35		50.58 **b**	45.97 **a**	2.20 **b**
PL-CONTR	MACRO	absent	absent	absent	100.00	26.85	58.32	14.83
	dev	-	-	-	*75.42*	*28.00*	*29.32*	*18.10*
PL-CONTR	MESO	absent	absent	absent	100.00	55.05	44.95	absent
	dev	-	-	-	65.36	*32.68*	*32.68*	-
mean			-	-		40.95 **bc**	51.63 **a**	14.83 **a**
PL-FERT	MACRO	absent	absent	absent	100.00	53.91	34.68	11.41
	dev	-	-	-	*62.45*	*28.43*	*23.58*	*10.44*
PL-FERT	MESO	absent	absent	absent	100.00	67.43	32.57	absent
	dev	-	-	-	*53.05*	*26.52*	*26.52*	-
mean						60.67 **ab**	33.63 **ab**	11.41 **b**
PL-GRAN	MACRO	absent	absent	absent	100.00	58.74	33.00	8.26
	dev	-	-	-	*52.60*	*23.95*	*24.16*	*4.48*
PL-GRAN	MESO	absent	absent	absent	100.00	77.88	22.12	absent
	dev	-	-	-	*52.96*	*26.48*	*26.48*	-
mean						68.31 **a**	27.56 **b**	8.26 **b**
Site		ns	ns	ns	ns	**	*	*
Aggregate fraction		ns	ns	ns	ns	**	ns	ns
Site X aggregate fraction		ns	ns	ns	ns	ns	ns	ns

Table 19. Organic matter form (organs and amorphous organic matter) distribution. The organs were further distinguished in strongly and moderately decomposed organs according to the degree of decomposition of the organic residues. The amorphous forms were distinguished on the basis of their colour (black, red and yellow). Numbers in italics show the standard deviation values. The letter show the significant difference and results of the two-away ANOVA calculated on the mean values (*: $p<0.05$, **: $p<0.01$, ns: not significant).

7.2 Qualitative assessment of organic matter composition

The O/C molar ratios of organic matter features were presented in Table 20. The checking of O/C of blank resin had been performed prior to carry out the elemental analysis of organic matter in macro- and mesoaggregates, in order to be confident that organic features other than resin had been analysed. The mean O/C ratio of blank resin was 0.05±0.02 (n=50), and it was significant lower ($p<0.05$) than the O/C ratio determined for any other organic features. The morphologically recognised organic forms showed a trend in the O/C ratio, which significantly increased ($p<0.05$), in each site, from organs (both moderately and strongly decomposed organs) and yellow amorphous forms to the red and black ones (Table 20). Regardless of aggregate classes, for the moderately decomposed organs, we found O/C values of 0.15 in the oak wood and 0.18 in the alfalfa which reached values of 0.43 and 0.49 for the black amorphous forms, respectively. In the walnut control, fertirrigate and granular, we found values from 0.57, 0.52 and 0.64 for the yellow amorphous forms to 1.04, 1.04 and 1.07 for the black amorphous organic matter forms. For both red and black amorphous organic forms, i.e. the two organic forms which had been found both in macro- and mesoaggregates in all sites (Table 19), we observed that the highest O/C was in macroaggregates in the case of oak, walnut control and fertirrigate, while the highest values of O/C ratio was in mesoaggregates for alfalfa and walnut granular (Table 20).

For each organic forms, the values of Fe/C, Al/C and Ca/C molar ratio had been determined in order to obtain a data reflecting the interaction of organic matter forms with the mineral phase, and on the average, significant differences in the values of molar ratio had been found in each site (Table 21). The values of Fe/C molar ratio were in fact higher in black amorphous forms than in the other forms in all sites. The same occurred for Al/C molar ratio in oak, alfalfa and walnut granular site, and for Ca/C molar ratio in oak and alfalfa sites (Table 21).

Organic form		Aggregate fraction	n	MO-W	MO-A	PL-CONTR	PL-FERT	PL-GRAN
Amorphous organic matter	Black	MACRO	174	0.43 **aA**	0.49 **aB**	1.04 **aA**	1,04 **aA**	1.07 **aB**
		dev		*0.05*	*0.05*	*0.04*	*0,03*	*0.01*
		MESO	136	0.40 **aB**	0.58 **aA**	0.93 **aB**	1.00 **aB**	1.14 **aA**
		dev		*0.04*	*0.03*	*0.03*	*0,01*	*0.08*
Amorphous organic matter	Red	MACRO	194	0.21 **bA**	0.35 **bB**	0.94 **bA**	0.97 **bA**	0.79 **bB**
		dev		*0.02*	*0.01*	*0.03*	*0.04*	*0.06*
		MESO	156	0.20 **bB**	0.39 **bA**	0.81 **bB**	0.83 **bB**	0.82 **bA**
		dev		*0.05*	*0.01*	*0.07*	*0.07*	*0.01*
Amorphous organic matter	Yellow	MACRO	100	0.16 **c**		0.57 **c**	0.52 **c**	0.64 **b**
		dev		*0.01*		*0.06*	*0.04*	*0.07*
		MESO	50	0.16 **c**	0.30 **c**			
		dev		*0.01*	*0.02*			
Organs	Strongly decomposed	MACRO	65	0.15 **c**	0.22 **c**			
		dev		*0.01*	*0.03*			
		MESO	30	0.15 **c**				
		dev		*0.01*				
Organs	Moderately decomposed	MACRO	27	0.15 **c**	0.18 **c**			
		dev		*0.01*	*0.01*			
		MESO						
		dev						

Table 20. The O/C molar ratio of organic features. The lowercase letters show the significant differences among organic matter forms and the uppercase letters show the significant different between macro and mesoaggregates at p level <0.05 Tukey test.

Organic form		Aggregate fraction	MO-W			MO-A			PL-CONTR			PL-FERT			PL-GRAN		
			Al:C	Fe:C	Ca:C	Al:C	Fe:C	Ca:C	Al:C	Fe:C	Ca:C	Al:C	Fe:C	Ca:C	Al:C	Fe:C	Ca:C
Amorphous organic matter	Black	MACRO	0.019 ***a***	0.006 ***a***	0.017 ***a***	0.022 ***a***	0.007 ***a***	0.023 ***a***	0.067 ***b***	0.029 ***a***	0.005	0.068	0.034 ***a***	0.004	0.084 ***a***	0.031 ***a***	0.004
		dev	*0.007*	*0.003*	*0.004*	*0.011*	*0.004*	*0.025*	*0.003*	*0.008*	*0.002*	*0.019*	*0.007*	*0.001*	*0.012*	*0.012*	*0.001*
		MESO	0.019 ***a***	0.006 ***a***	0.012 ***a***	0.040 ***a***	0.014 ***a***	0.017 ***a***	0.063 ***b***	0.059 ***a***	0.004	0.088	0.014 ***a***	0.004	0.094 ***a***	0.093 ***a***	0.002
		dev	*0.003*	*0.001*	*0.003*	*0.002*	*0.005*	*0.004*	*0.028*	*0.018*	*0.002*	*0.010*	*0.008*	*0.001*	*0.005*	*0.006*	*0.002*
Amorphous organic matter	Red	MACRO	0.005 ***b***	0.002 ***b***	0.007 ***b***	0.021 ***b***	0.006 ***b***	0.014 ***b***	0.080 ***a***	0.055 ***b***	0.005	0.094	0.044 ***a***	0.005	0.067 ***b***	0.023 ***b***	0.003
		dev	*0.001*	*0.000*	*0.001*	*0.008*	*0.002*	*0.003*	*0.015*	*0.020*	*0.001*	*0.010*	*0.006*	*0.001*	*0.022*	*0.005*	*0.001*
		MESO	0.009 ***b***	0.003 ***b***	0.007 ***b***	0.019 ***b***	0.006 ***b***	0.015 ***b***	0.066 ***a***	0.021 ***b***	0.003	0.064	0.016 ***a***	0.004	0.062 ***b***	0.058 ***b***	0.003
		dev	*0.008*	*0.003*	*0.002*	*0.000*	*0.001*	*0.001*	*0.020*	*0.006*	*0.000*	*0.005*	*0.013*	*0.002*	*0.010*	*0.006*	*0.001*
Amorphous organic matter	Yellow	MACRO	0.003 ***b***	0.001 ***b***	0.006 ***b***				0.045 ***c***	0.015 ***b***	0.004	0.040	0.015 ***b***	0.001	0.063 ***b***	0.011 ***c***	0.003
		dev	*0.001*	*0.000*	*0.001*				*0.005*	*0.006*	*0.003*	*0.010*	*0.001*	*0.001*	*0.004*	*0.000*	*0.002*
		MESO	0.005 ***b***	0.002 ***b***	0.005 ***b***	0.012 ***c***	0.003 ***c***	0.007 ***c***									
		dev	*0.002*	*0.001*	*0.002*	*0.002*	*0.001*	*0.000*									
Organs	Strongly decomposed	MACRO	0.003 ***b***	0.001 ***b***	0.003 ***c***	0.010 ***c***	0.003 ***c***	0.006 ***c***									
		dev	*0.000*	*0.000*	*0.001*	*0.007*	*0.001*	*0.001*									
		MESO	0.003 ***b***	0.001 ***b***	0.003 ***c***												
		dev	*0.000*	*0.000*	*0.001*												
Organs	Moderately decomposed	MACRO	0.003 ***b***	0.001 ***b***	0.003 ***c***	0.009 ***c***	0.002 ***c***	0.003 ***c***									
		dev	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*									
		MESO															
		dev															

Table 21. The Al/C, Fe/C and Ca/C molar ratio of organic forms. The letters show the significant different between organic form at p level <0.05 Tukey test.

7.3 Discussion

The microstructure features studied allowed us to deepen and better understand the results obtained in previous chapters (5 and 6). Briefly, from the data presented in chapters 5 and 6 we found that macro- and mesoaggregates were differently affected by soil management and aggregate genesis. In particular, the aggregation process seemed to have more effect on C dynamics in meso- than in macroaggregates, while the opposite occurred for the effect of soil management on the aggregation process. This latter statement is in agreement with Tisdall and Oades, (1980 and 1982), who reported that macroaggregates (2mm to 250 μm) were markedly affected by soil management. The behaviour of the mesoaggregates we investigated (1 mm to 250 μm) contrasted with those previous results.

The data obtained by aggregate thin section investigation, however, confirmed that macro- and mesoaggregates differed. They were in fact physically differentiated microhabitats, as shown by the porosity and distribution of pores as determined by image analysis. Mesoaggregates were more compact (less porous) than macroaggregates, probably because of the lack of the largest pore classes (i.e., 100-200 and 200-350 μm) which were detected in the macroaggregates. This was in agreement with Dexter (1988) who stated that smaller size aggregates were higher in density than larger aggregates, because of the hierarchical pore exclusion principle. According to this, with each smaller size of aggregates, the pore space within the immediately greater aggregate size is excluded. Thus, pores >100 μm will be excluded in mesoaggregates (with a mean size of 0.55-0.65 mm). In contrast to this principle, we found that mesoaggregates were more porous than macroaggregates when porosity was measured by Hg intrusion porosimetry (see ch. 5 and 6). But, as shown by the pore size distribution, the differences in porosity measured by Hg intrusion related to particle size distribution (i.e., textural porosity, Le Bissonnais and Attou, 1998; Elliott and Coleman, 1988; Hassink et al., 1993; Jiang et al. 2011). By contrast, optical observation allows one to investigate the structural porosity (Pagliai et al.1988, Li et al., 2004). Thus, we concluded that the porosity in the mesoaggregates we investigated entailed a limitation on C dynamics for at least two reasons: i) lower structural porosity due to porosity-based exclusion of large transmission pores (pores >100 μm) able to maintain good soil structure condition and soil drainage (Greenland, 1977), and ii) higher textural porosity because of finer particle size which corresponds to textural pores originating from clay-clay interactions and related therefore to the amount of clay particles.

The hypothesis that mesoaggregates area microhabitat enabling organic matter conservation, due to a slowing down of organic matter turnover, seemed to be confirmed by the amount of organic

forms. Mesoaggregates were richer in organic forms than macroaggregates. Moreover, the amount of organic matter correlated with the organic C content measured on aggregates by dry combustion, confirming that the amount of organic forms morphologically defined by image analysis, was related to soil organic matter content.

The interaction between site and aggregate size was, however, significant for the amount of organic forms, and accordingly the mesoaggregates of oak and alfalfa were the richest in organic forms. This suggested that soil management cannot be excluded as a factor affecting C dynamics in mesoaggregates. Aggregate genesis created a physical habitat which, coupled with differences in quantity and/or quality of organic matter input, defined the organic matter turnover. Chenu et al., (2001) and Negassa et al., (2015) asserted that the accessibility of organic matter in the soil microbial community (substrate availability) is determined by pore distribution, which also determines water potential and the oxygen flux. Thus the great amount of organic matter observed in mesoaggregates may be less available to microorganisms, as this class of aggregates is limited by a pore size distribution characterized by lack of pores >100 um.

If a large proportion of intra-aggregate organic matter is occluded so that microbes cannot utilize it, there will be fewer interfaces between organic matter and pores (i.e., with an expected low values of exposure index, EI). In our study the EI provides us with important information because in all sites we observed that macroaggregates have more occluded organic matter (i.e., lower EI values), which is potentially less available for the microbial community than in mesoaggregates. This was apparently in disagreement with the well-known theory of organic matter occlusion and stabilization occurring in smaller aggregates rather than in larger ones (Rabbi et al., 2014). From our data we can enrich this concept, thanks to the correlation between EI and qualitative data relating to organic matter measured on aggregates (i.e., C/N ratio, $\delta^{13}C$, enzyme activity). First of all, one needs to take into account that the exposure index provides information on the exposure of organic matter to the pores interface, which corresponds to a potential availability of organic matter, but not to a mandatorily higher organic matter transformation. From the positive relationship between EI and C/N ratio, we observed that the EI did not coincide with loss of organic C. The C/N ratio is a simple and rather common indicator of the whole organic matter pool turnover (Bronick and Lal, 2005), and an increase in the C/N ratio suggests a conservative C process. The negative correlation between EI and $\delta^{13}C$ confirmed this. The value of $\delta^{13}C$ was another useful index of the degree of transformation and of the relative stability of the organic substance of a soil. During the processes of decomposition of organic substance there occurs isotopic carbon fractionation.

^{12}C is rapidly oxidized by microorganisms, leading to an enrichment in ^{13}C. It follows that higher values of $\delta^{13}C$ (positive) correspond to a more transformed organic substance (Angers et al., 1997). Such qualitative information on organic matter (C/N and $\delta^{13}C$), combined with the higher EI values in mesoaggregates, suggested that, even though the organic compounds were more exposed in mesoaggregates, this corresponded to a lower organic matter transformation. And this was the effect of physical occlusion. Mesoaggregates thus showed organic matter that was potentially more available as it was close to pores, but physically inaccessible to microbial biomass due to lower porosity and smaller pore size distribution (as discussed above) than in macroaggregates. One possible explanation of why EI had higher values in mesoaggregates than in macroaggregates may be related to the aggregation process and to the role that organic residue transformation had on the formation of smaller aggregates within macroaggregates. In fact, new smaller aggregates form around particulate organic matter encrusted with microbial products (e.g., Six et al., 2000; Six et al., 2004). This means that organic matter must be accessible to microorganisms in order for smaller aggregates to forms, and hence for organic matter to be close to pores.

With regard to the distribution of organic forms, the site was a factor that affected both organs and amorphous organic matter. First of all, in oak and alfalfa we observed the presence of both organ forms. The occurrence of both organs and amorphous organic matter in oak and alfalfa suggested that amorphous organic matter could be the end-product of organic residues transformation, and therefore it would probably be less labile than the initial C forms (Falsone et al., 2014). Additionally, oak had a higher amount of organs than the alfalfa site, which could be due to the different type of organic matter between the two sites. In chapter 5 we reported that organic matter from the alfalfa site was possibly richer in cellulose and poorer in lignin and this would allow for easier alteration of the organic matter. We must also add that the alfalfa site had more nitrogen than the oak wood site. Consequently it was possible that organs from alfalfa site have been more degradable and therefore less present. In the case of the walnut sites, the lack of organs was probably due to from the presence of more transformed organic matter (chapter 6) and probably linked to the mineral phase of the soil.

In the case of amorphous organic forms we observed that black forms generally prevailed, and among walnut sites, where organs were not found, the black amorphous forms prevailed in N fertilized sites (both fertirrigated and granular) more than in the walnut control. Black amorphous forms also prevailed in mesoaggregates, and they were the only organic forms affected by the aggregate size. The effect of aggregate size on black amorphous organic matter was independent from sites, because the interaction site x aggregates fraction was not significant. These results

suggested that the black amorphous forms were the most stable organic forms protected by both physical occlusion in smaller aggregates (mesoaggregates) and by chemical recalcitrance, being the end-product of soil organic matter transformation.

As soil organic matter decomposes, it becomes depleted in labile fractions, and the most recalcitrant C remains; therefore, we thus expected a variation in C stability related to organic features described through micromorphology (Blazejewski et al., 2005). Element analysis supported this hypothesis. According to the site, a clear trend in the O/C ratio was found: moderately, strongly decomposed organs and yellow amorphous organic forms had similar mean O/C ratios; red amorphous organic matter had significantly higher mean O/C ratio than organs and yellowish features, while black amorphous forms had the highest O/C ratio. This criterion (O/C ratio) appeared valid to discriminate among organic features. Moreover, higher values of the O/C ratio were expected in the amorphous features as a result of SOM transformation processes (Haumaier and Zech, 1995), thus supporting the finding that the black amorphous forms were the most transformed organic forms. As regards the absolute values of the O/C ratio in the different sites and organic forms, we must take into account that, besides being influenced by the increase in oxidation of organic matter during the decomposition and humification processes, the O/C ratios also closely strongly depend on the source of the organic residue. For instance, the O/C ratio reported for lignin and cellulose, two of the most abundant biopolymers in the soil (Kögel-Knabner, 2002), were 0.37 and 0.83 (Stoffyn-Egli et al., 1997). As visible from the data reported in table 20, the O/C ratios in oak were in general lower than those from corresponding organic forms in the alfalfa site. And, O/C ratios in the alfalfa site were lower than those in walnut sites. Thus, a clear effect from the organic residue origin may be supposed. However, the values > 1.0 observable in black and red amorphous organic matter in the walnut sites may be due to greater interaction with the mineral phase. SEM-EDS analysis was a punctual analysis, so the values of the elemental composition of both organic substance and mineral phase (e.g., silicates, oxides) interacting with the organic compounds was detected.

Elements such as Al, Fe and Ca were definitely related to the mineral phase. Significant differences in mean Al/C, Fe/C and Ca/C molar ratio were found. The Al/C and Fe/C molar ratios were in general higher in walnut sites than in oak and alfalfa, thus confirming that higher mineral-organic matter interaction occurred in walnut sites. The differences in the Al/C, Fe/C and Ca/C molar ratios provided further differentiation between organs and amorphous features in all sites. Amorphous black organic matter in fact showed the highest Al/C and, Fe/C, but also the highest Ca/C in the

case of oak and alfalfa sites. These data suggested that some interactions between amorphous features and mineral particles, likely Al (silicates) and Fe (hydr) oxides and Ca, have occurred. Consequently, these organic features could be further stabilized due to binding to minerals and organic and inorganic interactions (Brown et al., 2000). Hence, the black amorphous forms were the most oxidized (high O/C molar ratio), the most interacting the mineral phase (high Al/C, Fe/C and, at least in certain cases, Ca/C molar ratios) and the prevailing organic forms, especially in mesoaggregates. This corresponds to higher stabilization of C due to mineral interaction, chemical recalcitrance and physical occlusion (Sollins et al., 1996).

7.4 Conclusions

The hypothesized differences in the features of organic matter, due to physical location and the spatial relationship between organic compounds and pores have been confirmed. Our data show that macro- and mesoaggregates were physically differentiated microhabitats. The mesoaggregates showed organic matter that was potentially more available as it was near to the pores, but physical inaccessible to microbial biomass due to lower porosity and smaller pore size distribution than in macroaggregates. The proximity of organic matter to the pores should be the origin of mesoaggregates, whose genesis begins thanks to organic residue decomposition leading to organic matter encrusted with microbial products. The aggregation process therefore appears as a C-dissipative process, at least in the first step of mesoaggregate formation. In “mature” mesoaggregates, on the other hand, the organic C was strongly stabilized, because of the occurrence of several processes. The prevalence of black amorphous organic matter as the organic form indicates in fact the prevalence of organic matter that is i) chemically recalcitrant, as suggested by higher O/C values, and ii) strongly interacting with the mineral phase, as indicated by high Al/C, Fe/C and, at least in certain cases, Ca/C molar ratios. Additionally, this particularly occurs in mesoaggregates, iii) where porosity and pore size distribution limit accessibility of organic matter to microbial biomass. This corresponds to high stabilization of C due to chemical recalcitrance (i), mineral interaction (ii) and physical occlusion (iii).

8. GENERAL CONCLUSIONS

This interdisciplinary research on soil aggregates allowed us to evaluate the effect of management on biophysical properties of different aggregate size classes, and to verify the assumption that different aggregate fractions represented different microhabitats each of which had specific biophysical properties affecting soil functionality (e.g., soil carbon sequestration function) in both agroecosystems investigated.

In the mountain agroecosystem we observed the effect of the organic matter quality in all aggregate fractions (different litter between oak wood and alfalfa sites) and the physical architecture of the aggregates. The macroaggregates of oak wood were characterized by higher accumulation of organic C but not very available for microorganisms. In the oak wood, both the quality of organic matter and aggregate porosity contributed to creating a non-favourable habitat for microbial activity despite the greater input of organic C than in alfalfa. On the contrary, as was evident from the interaction between the enzymatic and biological properties, of the macro- and mesoaggregates, in the alfalfa site we found a better habitat for microbial activity and functional diversity with positive effects on C storage in the soil, due also to the organic matter quality (litter).

The biophysical parameters studied also provided important information on aggregate fraction behaviours. Thus, in the macrohabitat it was been possible to attribute the low microbial efficiency observed in the oak wood site to the quality of C input (probably a higher presence of lignin and lower C/N ratio) and to the effect of higher fine porosity (due to higher presence of clay) in this aggregate fraction. The study of the meso- and microhabitats confirmed that the aggregate formation process produces smaller aggregates rich in transformed organic molecules. Mesoaggregates (aggregates between 1 mm and 250 μm) behave more like microaggregates, and therefore are much more influenced by the aggregative process than by management.

In the plain agroecosystem, no effect was produced by urea addition to the organic matter content in any aggregate fraction, probably due to the low quality of fertilizer used. Urea addition however had direct (on enzyme activity of C –Cycle) and indirect effects (i.e. through root development) on the quality of the organic matter. In macroaggregates the higher value of labile C attributable to root growth in fertilized sites promoted enzymatic activity and the physical occlusion while limiting the degree of organic matter transformation. Walnut fertirrigate and granular sites showed a habitat with greater total volume of porosity and higher abundance of the small pore class which made for physical protection of organic matter, a further reason limiting microbial activity and, consequently, degradation of organic matter in walnut fertirrigate and granular sites.

The effect of urea addition decreases with the decrease in the size of the aggregates. Only in the mesoaggregates of granular and fertirrigates walnut sites was it possible to observe an increase in the specific enzymatic activity of β-glucosidase, but it was related to the particle distribution rather than N addition. Once again we observed how mesoaggregates were not much influenced by different styles of management.

Briefly, from the data of mountain and plain agroecosystem we found that macro- and mesoaggregates were differently affected by soil management and aggregates genesis. In particular, the aggregation process seemed more relevant to C dynamics in meso- than in macroaggregates, while the opposite occurred for soil management. Whereas Tisdall and Oades, (1980 and 1982) reported that macroaggregates were strongly affected by soil management, the behaviour of our investigated mesoaggregates (1 mm to 250 μm) conflicted with that judgment. To Tisdall and Oades (1982), microaggregates (<250 μm) were less influenced by management. From our study, by contrast that limit needed to be moved to at least 1 mm, at least for the management that did not cause pronounced disturbance to aggregation, as in oak wood, alfalfa and walnut sites.

The data obtained by aggregate thin section investigation confirmed that macro- and mesoaggregates were different habitats. As further confirmation of this we had the evidence that the physical location of organic matter and the spatial relationship between organic matter and pores were different in the two aggregate classes (macro and mesoaggregates) and, as a consequence, the features of organic matter (i.e., morphological form and chemical composition) differed. In particular, mesoaggregates had a higher amount of transformed organic forms (i.e., amorphous forms) and were potentially more available, as they were more exposed to the pore surface, being near the pores, but were physical inaccessible to microbial biomass due to lower porosity and smaller pore size distribution than in macroaggregates. We hypothesized that the high surface of organic matter exposed to pores in mesoaggregates was due to the aggregation process and to the role that organic residue transformation plays in the formation of smaller aggregates within macroaggregates. In mesoaggregates the black amorphous form prevailed and this form was the most stable organic form protected by both physical occlusion in smaller aggregates (mesoaggregates) and chemical recalcitrance because it is the end-product of soil organic matter transformation. This las point was confirmed by higher O/C values. What is more, it was the most interactive with the mineral phase (high Al/C, Fe/C and, at least in certain cases, Ca/C molar ratios). This corresponds to higher stabilization of C due to mineral interaction, chemical recalcitrance and physical occlusion.

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10. SUPPLEMENTARY MATERIAL

Site	Aggregate size fraction	C g kg^{-1}	N g kg^{-1}	Al g kg^{-1}	Fe g kg^{-1}	CaCO3 g kg^{-1}	C_{mic} mg kg^{-1}	N_{mic} mg kg^{-1}	C_{extr} mg kg^{-1}	N_{extr} mg kg^{-1}
MO-W	MACRO	44.7	3.86	37.88	22.24	93.3	306.3	56.3	288.0	116.1
		(1.6)	*(0.13)*	*(0.46)*	*(0.45)*	*(13.3)*	*(3.2)*	*(6.0)*	*(1.2)*	*(0.3)*
	MESO	49.6	4.10	38.19	22.64	67.6	266.1	48.3	240.9	108.0
		(1.8)	*(0.27)*	*(0.39)*	*(0.25)*	*(16.6)*	*(1.0)*	*(1.2)*	*(8.1)*	*(0.5)*
	MICRO	51.4	4.16	38.40	23.33	70.9	220.8	52.4	208.3	83.5
		(1.2)	*(0.06)*	*(2.70)*	*(0.71)*	*(11.8)*	*(13.4)*	*(2.4)*	*(11.3)*	*(3.4)*
MO-A	MACRO	10.9	1.06	22.39	16.41	98.5	134.8	18.3	110.8	48.5
		(0.6)	*(0.22)*	*(1.30)*	*(0.40)*	*(16.3)*	*(2.6)*	*(0.2)*	*(0.5)*	*(4.2)*
	MESO	12.1	1.14	25.01	16.59	87.9	126.1	10.8	73.8	40.8
		(0.6)	*(0.24)*	*(0.65)*	*(0.16)*	*(7.5)*	*(4.4)*	*(0.9)*	*(4.9)*	*(7.4)*
	MICRO	13.2	1.25	26.46	19.48	77.4	99.6	13.4	71.7	46.7
		(1.0)	*(0.21)*	*(1.31)*	*(0.17)*	*(1.2)*	*(11.9)*	*(2.6)*	*(4.2)*	*(5.7)*
PL-CONTR	MACRO	6.6	0.82	27.97	20.96	-	50.3	5.9	114.4	16.8
		(1.8)	*(0.16)*	*(2.60)*	*(1.89)*		*(7.1)*	*(2.0)*	*(4.9)*	*(2.8)*
	MESO	7.8	0.90	30.95	22.15	-	52.4	6.1	99.0	15.3
		(1.2)	*(0.13)*	*(4.66)*	*(2.26)*		*(6.8)*	*(2.8)*	*(2.5)*	*(2.4)*
	MICRO	6.8	0.83	27.01	19.50	-	34.2	8.0	84.7	11.9
		(0.9)	*(0.08)*	*(6.48)*	*(2.47)*		*(4.3)*	*(0.8)*	*(0.1)*	*(1.9)*
PL-FERT	MACRO	7.9	0.95	33.17	22.74	-	72.3	10.0	103.8	14.7
		(1.2)	*(0.11)*	*(2.97)*	*(0.35)*		*(5.0)*	*(1.0)*	*(2.5)*	*(0.2)*
	MESO	9.3	1.03	33.58	23.35	-	70.5	7.1	92.2	14.3
		(3.0)	*(0.23)*	*(3.13)*	*(0.51)*		*(7.8)*	*(0.5)*	*(2.1)*	*(2.0)*
	MICRO	8.2	0.97	31.22	21.23	-	58.7	5.9	98.6	17.1
		(1.8)	*(0.15)*	*(3.32)*	*(0.03)*		*(1.7)*	*(0.6)*	*(2.1)*	*(0.5)*
PL-GRAN	MACRO	8.8	1.01	32.62	23.12	-	73.4	10.0	106.9	16.3
		(1.5)	*(0.08)*	*(7.28)*	*(2.86)*		*(1.1)*	*(0.1)*	*(4.1)*	*(1.7)*
	MESO	8.6	1.00	31.42	23.79	-	71.7	9.2	106.6	15.2
		(0.8)	*(0.00)*	*(9.75)*	*(3.97)*		*(0.8)*	*(0.1)*	*(0.1)*	*(2.5)*
	MICRO	8.3	0.98	29.19	21.31	-	46.0	5.7	101.7	14.7
		(0.2)	*(0.07)*	*(11.07)*	*(4.43)*		*(5.2)*	*(1.8)*	*(6.8)*	*(2.6)*

Table 22. Main chemical and biological characteristics of the aggregates fraction. The numbers in parentheses and italic are standard deviation values.

Site	Aggregate size fraction	Extracellular enzymatic activity nmol MUF h^{-1} g^{-1}							
		β-GLU	α-GLU	N-AG	β-XYL	β-CEL	SULF	PME	PDE
MO-W	MACRO	472.9	19.1	305.4	54.6	80.1	504.3	557.8	219.1
		(1.3)	*(3.0)*	*(3.0)*	*(5.2)*	*(4.8)*	*(0.4)*	*(7.9)*	*(27.4)*
	MESO	419.5	16.7	325.1	54.3	76.4	608.1	706.1	206.7
		(11.8)	*(1.3)*	*(24.5)*	*(7.5)*	*(1.8)*	*(0.6)*	*(14.0)*	*(13.7)*
	MICRO	547.4	20.5	367.5	70.6	86.4	651.2	756.7	236.3
		(15.7)	*(2.1)*	*(16.6)*	*(0.2)*	*(8.7)*	*(21.1)*	*(2.6)*	*(2.5)*
MO-A	MACRO	229.4	8.3	70.7	28.2	52.2	89.8	126.4	57.3
		(74.6)	(2.7)	(13.5)	(4.6)	(5.4)	(4.6)	(13.6)	(7.6)
	MESO	278.4	11.1	85.8	40.6	56.4	104.4	173.5	71.4
		(20.0)	(3.3)	(6.3)	(7.5)	(7.8)	(6.8)	(12.2)	(2.4)
	MICRO	348.2	13.7	108.2	61.3	86.0	140.5	187.9	89.3
		(28.0)	(3.1)	(12.5)	(6.7)	(9.5)	(16.2)	(4.5)	(10.7)
PL-CONTR	MACRO	86.9	4.7	26.3	14.5	8.2	74.4	131.0	36.8
		(1.5)	(0.6)	(10.3)	(4.5)	(2.3)	(7.7)	(11.4)	(7.6)
	MESO	83.1	5.4	42.0	17.8	9.6	92.1	154.4	45.4
		(8.6)	(0.8)	(7.8)	(4.3)	(3.3)	(1.6)	(0.2)	(7.8)
	MICRO	91.3	6.1	41.0	19.4	9.4	98.1	162.9	51.9
		(1.1)	(1.1)	(11.7)	(4.6)	(2.8)	(1.5)	(19.1)	(10.8)
PL-FERT	MACRO	97.3	8.3	74.9	23.2	20.0	64.8	184.8	50.0
		(6.7)	*(0.9)*	*(0.9)*	*(6.5)*	*(0.8)*	*(4.2)*	*(15.6)*	*(6.1)*
	MESO	147.2	9.1	56.2	34.5	20.2	82.7	183.0	65.2
		(9.5)	*(1.6)*	*(3.9)*	*(5.8)*	*(7.1)*	*(8.2)*	*(27.2)*	*(0.7)*
	MICRO	126.7	8.3	65.1	27.3	21.1	84.5	225.6	56.6
		(13.0)	*(2.1)*	*(8.0)*	*(8.0)*	*(1.1)*	*(8.9)*	*(20.2)*	*(11.6)*
PL-GRAN	MACRO	146.6	6.0	54.4	26.4	25.8	101.7	206.6	60.1
		(7.4)	*(0.2)*	*(17.6)*	*(2.3)*	*(7.8)*	*(1.2)*	*(3.4)*	*(13.0)*
	MESO	112.4	8.3	58.7	36.7	24.2	101.5	215.1	61.5
		(1.7)	*(1.9)*	*(7.9)*	*(0.9)*	*(5.1)*	*(7.3)*	*(15.1)*	*(0.0)*
	MICRO	172.5	9.1	63.6	25.1	24.0	117.7	206.0	63.0
		(5.9)	*(1.8)*	*(2.0)*	*(5.4)*	*(3.7)*	*(7.5)*	*(6.0)*	*(3.8)*

Table 23. Enzymatic activities of the aggregates fraction β-glucosidase (β-GLU), α-glucosidase (α-GLU), N-acetyl β-glucosaminidase (N-AG), β-xylosidase (β-XYL), β-cellobiosidase (β-CEL), Arylsulfatase (SULF), Phosphomonoesterase (PME) and Phosphodiesterase (PDE) in macro-, meso- and microaggregates. The numbers in parentheses and italics are standard deviation values.

Site	Aggregate size fraction	DNA $_{tot}$ g µg $^{-1}$	H'	J'
MO-W	MACRO	29.2 *(13.5)*	0.49	0.82
	MESO	25.9 *(13.6)*	0.60	0.84
	MICRO	60.0 *(32.8)*	0.64	0.72
MO-A	MACRO	12.6 *(5.9)*	0.71	0.83
	MESO	20.6 *(7.5)*	0.83	0.90
	MICRO	23.7 *(6.6)*	0.73	0.88
PL-CONTR	MACRO	4.0 *(1.1)*	0.84	0.86
	MESO	8.7 *(0.2)*	0.71	0.81
	MICRO	9.0 *(2.7)*	0.96	0.91
PL-FERT	MACRO	5.4 *(0.5)*	0.95	0.85
	MESO	6.2 *(3.7)*	1.04	0.96
	MICRO	10.9 (3.8)	0.88	0.92
PL-GRAN	MACRO	6.6 *(2.1)*	0.93	0.80
	MESO	11.8 *(3.1)*	0.89	0.88
	MICRO	8.8 *(3.5)*	0.85	0.85

Table 24. Amounts of DNA extraction efficiency, Shannon Winer (H') and Eveness (J') index in the aggregates fraction. The numbers in parentheses and italic are standard deviation values.

Site	Aggregate size fraction	Clay (<2 μm) g kg^{-1}	Total Sand (2-0.05 mm) g kg^{-1}	Coarse Sand (2-0.2 mm) g kg^{-1}	V_{Hgtot} mm^3 g^{-1}	SSA_{tot} m^2 g^{-1}
MO-W	MACRO	42.72	37.83	5.07	171.41	8.34
		(1.73)	*(2.60)*	*(0.03)*	*(17.89)*	*(0.50)*
	MESO	33.84	32.26	5.14	218.36	8.58
		(3.60)	*(4.64)*	*(1.28)*	*(12.38)*	*(2.28)*
	MICRO	41.26	20.38	0.60	209.94	8.83
		(0.68)	*(3.47)*	*(0.04)*	*(9.84)*	*(0.69)*
MO-A	MACRO	16.23	51.06	23.44	126.68	1.87
		(6.17)	*(0.54)*	*(1.90)*	*(7.54)*	*(0.40)*
	MESO	24.67	51.57	24.87	207.52	3.43
		(4.16)	*(0.22)*	*(3.98)*	*(13.32)*	*(0.04)*
	MICRO	25.75	36.92	0.48	131.78	1.94
		(4.98)	*(4.47)*	*(0.12)*	*(11.53)*	*(0.78)*
PL-CONTR	MACRO	224.40	399.14	38.37	130.79	4.04
		(58.74)	*(65.93)*	*(16.59)*	*(8.86)*	*(0.11)*
	MESO	237.75	391.52	49.27	223.96	2.14
		(35.38)	*(48.71)*	*(18.28)*	*(36.37)*	*(0.29)*
	MICRO	229.44	397.50	1.02	125.41	0.63
		(48.19)	*(24.84)*	*(0.02)*	*(1.05)*	*(0.04)*
PL-FERT	MACRO	236.94	393.90	35.03	147.10	4.48
		(12.40)	*(15.26)*	*(7.71)*	*(1.42)*	*(0.40)*
	MESO	262.68	357.14	48.36	237.69	4.48
		(6.10)	*(19.00)*	*(12.34)*	*(35.81)*	*(0.25)*
	MICRO	229.98	387.76	1.02	161.50	4.07
		(10.74)	*(20.38)*	*(0.02)*	*(6.75)*	*(0.20)*
PL-GRAN	MACRO	233.53	376.24	34.70	144.40	3.67
		(81.91)	*(56.93)*	*(11.07)*	*(9.82)*	*(0.67)*
	MESO	258.28	359.39	42.71	203.02	1.82
		(68.31)	*(70.99)*	*(19.26)*	*(6.13)*	*(0.03)*
	MICRO	242.63	388.74	1.51	130.80	2.05
		(57.61)	*(65.70)*	*(0.68)*	*(5.67)*	*(0.12)*

Table 25. Main physical characteristics as clay, yotal sand, coarse sand and total volume of pore (V_{Hgtot}) and specific surface area of the pore (SSA_{tot}) of the aggregates fractions. The numbers in parentheses and italics are standard deviation values.

																				FORM								SURFACE										
			ABUNDANCE		TYPES PEDS	PEDALITY		TYPES VOIDS				TYPES ORGANIC MATTER			COLOR		ROUNDNESS				SPHERICITY				ROUGHNESS			SMOOTHNESS			ACCOMO		ORIENTATION	
MANAGEMENT	AGGREGATES		FREQUENCY	ABUNDANT	SPHEROIDAL	MODERATELY	WEAKLY	COUMPOUND	COMPLEX	CHANNELS	PLANES	CELLS	AMORPHOUS	PUNTUATION	RED	BLACK	ROUNDED	SOBROUNDED	SUBANGULAR	ANGULAR	TABULARE	OVOID	SUBSPHERICAL	SHERICAL	SERRATE	MAMMILATE	DIGITATE	ROUGH	UNDULATING	SMOOTH	PARTIALLY	UNACC	RANDOM	REPRESENTS
MO-W	MACRO	PEDS			*	*													*				*			*										
		VOIDS	10%	frequenty					* 6		*4								*				*			*				*			*		*	
		OM	30%	very abundant										*20	*10	*20	*							*		*			*				*		*	
	MESO	PEDS			*	*													*				*			*			*							
		VOIDS	5%	occasional				*5											*		*					*			*				*		*	
		OM	40%	very abundant										*40	*20	*20	*						*						*				*		*	
MO-A	MACRO	PEDS			*		*											*					*			*			*							
		VOIDS	30%	very abundant					*25	*5									*				*			*	*		*			*	*		*	
		OM	20%	abundant										*12	*8	*12	*							*		*				*			*		*	
	MESO	PEDS			*		*												*					*		*			*							
		VOIDS	5%	occasional				*5											*			*				*			*				*		*	
		OM	20%	abundant										*20		*20	*							*			*			*						*	
PL-CONTR	MACRO	PEDS			*		*												*				*			*			*							
		VOIDS	20%	abundant					*									*			*					*			*			*			*	
		OM	5-10%	less frequenty										*7		*7	*							*		*			*				*		*	
	MESO	PEDS			*		*													*				*		*			*							
		VOIDS	5%	occasional				*5												*	*					*			*				*		*	
		OM	10-15%	more frequenty										*15	*10	*5	*							*		*			*				*		*	
PL-GRAN	MACRO	PEDS			*	*													*				*			*			*							
		VOIDS	20%	abundant					*10	*10										*	*					*			*			*	*			
		OM	10-15%	more frequenty										*10	*5	*10	*		*			*				*			*				*		*	
	MESO	PEDS			*		*												*				*			*			*							
		VOIDS	5%	occasional				*5									*						*			*			*				*		*	
		OM	15-20%	less abundant										*5	*15	*5			*				*			*			*				*		*	
PL-FERT	MACRO	PEDS			*	*												*					*			*			*							
		VOIDS	10%	frequenty					*									*				*				*	*		*				*		*	
		OM	20-25%	more abundant										*5	*5	*20	*							*			*			*				*		*	
	MESO	PEDS			*		*												*					*		*			*							
		VOIDS	10%	frequenty				*												*				*			*			*				*		*	
		OM	20%	abundant										*20	*10	*10	*							*			*			*				*		*	

Table 26. Qualitative description of the thin section images taken by optical microscope, following guidelines recommended by Stoops (2003). The numbers with asterisk represented the percentage of type of voids, type of organic matter and colour of organic matter.

SITE	FRACTION	AREA AGGREGATES (mm²)	AREA POROSITY (mm²)	AREA SOM (mm²)	SOM-PORE mm
MO-W	MACRO	8.60	0.48	1.03	0.52
		(3.48)	*(0.18)*	*(0.63)*	*(0.34)*
MO-W	MESO	0.33	0.01	0.05	0.04
		(0.19)	*(0.01)*	(0.02)	*(0.02)*
MO-A	MACRO	3.23	0.30	0.24	0.13
		(1.71)	*(0.15)*	*(0.10)*	*(0.06)*
MO-A	MESO	0.24	0.02	0.03	0.03
		(0.16)	*(0.01)*	(0.03)	*(0.02)*
PL-CONTR	MACRO	11.35	0.70	0.44	0.06
		(6.82)	*(0.44)*	*(0.75)*	*(0.04)*
PL-CONTR	MESO	0.25	0.01	0.03	0.01
		(0.15)	*(0.00)*	*(0.02)*	*(0.01)*
PL-FERT	MACRO	7.15	0.47	0.38	0.11
		(3.20)	*(0.16)*	*(0.27)*	*(0.08)*
PL-FERT	MESO	0.23	0.01	0.02	0.01
		(0.16)	*(0.02)*	*(0.01)*	*(0.01)*
PL-GRAN	MACRO	6.23	0.52	0.29	0.08
		(4.54)	*(0.23)*	*(0.18)*	*(0.06)*
PL-GRAN	MESO	0.24	0.02	0.03	0.01
		(0.16)	*(0.01)*	*(0.02)*	*(0.01)*

Table 27: Main micromorpholy observation, image analysis of area aggregates, area pore, area organic matter and the perimeter of organic matter in contact to the pore (SOM-PORE). The numbers in parentheses and italics are standard deviation values.