University of Naples Federico II
Department of Agriculture

Joint international Ph.D. Italy - Chile in Environmental Resources Sciences
XXVIII Cycle (2013-2016)

Sustainability and impact on C storage and soil quality of different cropping systems and agricultural managements

Ph.D. Dissertation
by Elisa Niro

TUTOR:
Dott.ssa. Rosaria D'Ascoli

CO-TUTOR
Dott. Fabio Marzaioli

TUTOR:
Prof. María De la Luz Mora

COORDINATOR:
Prof. Maria A. Rao

Table of contents

Chapter 1- General introduction

1.1. Soil organic C stock

Terrestrial C pool, composed by soil and biota C, is a very important component of the global C cycle. (Le Queré 2008). The quantity of C stored in soil is second only to that in the oceans (38400 Gt), in particular the soil C pool is more than four time the amount of C in biota (~560 Gt of organic C) (Stockmann et al., 2013): approximately 2344 Gt of organic C is stored in the top three meters of soil, 1500 Gt in the first meter and about 615 Gt in the top 20 cm (Jobbágy & Jackson, 2000; Guo & Gifford, 2002). In the global C cycle, oceans and lands are considered a C sink, while the atmospheric CO_2 increase is caused by anthropogenic emissions of CO_2 due to fossil fuel burning (6,3 ±0,4 PgC y^{-1} during 1190 to 1999; Prentice et al., 2001), but the rate of increase of atmospheric CO_2 was about 3,3 ± 0,1 PgC y^{-1} and is less than the emission because some CO_2 dissolves into the oceans and some is taken up by terrestrial ecosystems. The uptake by terrestrial ecosystems is due to an excess of primary production (photosynthesis) over respiration and other oxidative processes (decomposition or combustion of organic matter) and the gross primary production (GPP) represents the CO_2 fixed from the atmosphere during photosynthesis (Prentice et al., 2001). It is estimate that about half of GPP is incorporated into new plant tissues and the other half is converted in CO_2 by autotrophic respiration, (Lloyd & Farquhar, 1996; Waring et al., 1998). Therefore the difference between photosynthesis and autotrophic respiration is the annual plant growth and is known as net primary production (NPP). The C fixed in NPP returns to atmospheric CO_2 pool through two process: heterotrophic respiration (Rh) by decomposers, herbivores and carnivores, and combustion (such as by fire) (Prentice et al., 2001). The difference between NPP and Rh determines how much C is lost or gained by the ecosystem in the absence disturbance that removes C from the ecosystem (as well as harvest or fire). This C balance known as net ecosystem production (NEP), can be estimated from changes in C stocks or by measuring the fluxes of CO_2 between land and

atmosphere. If other losses of carbon occur (harvest, fire, erosion, export of dissolved or suspended organic carbon), what remain is net biome production (NBP), for example, the C accumulated by the terrestrial biosphere (Schulze & Heimann, 1998) and finally, it represents the net land uptake on global scale.

In a steady state ecosystem, Rh and other C losses would equal the NPP, and NBP would be zero, but the action of human activities, natural disturbances, and climate variability affect NPP and Rh, causing a transient changes in terrestrial C pool and a non-zero NBP. If the rate of C input changes (NPP), also the C output changes (Rh), but there is a different time of response. In the global carbon cycle, terrestrial system acts as a carbon sink; while the likely mechanisms that produce the sink are known, their relative contribution are still uncertain (Prentice et al., 2001). Same processes or environmental variables that control the terrestrial or soil carbon sink are: natural climate variability and/or disturbance regime affecting NBP through NPP, chemical and physical properties of litter, stocks of living biomass, stocks of detritus and soil carbon, environmental controls on decomposition and rates of biomass removal. Moreover also the human activity play an important role in this balance by land use change and land management.

1.1.2. Land use and land use change effects on SOC stock

The soil C stock is determined by the balance between the net C inputs (as organic matter, OM) and the net C losses (as CO_2, dissolved organic carbon, loss through erosion) (Smith, 2007), that are influenced by biotic characteristics (such as biomass production and microbial abundance), environmental variables (e.g., mean annual precipitation and temperature), soil characteristics (including texture and lithology) and anthropogenic controls, like land use and management (Albaladejo et al., 2013). In fact C inputs are largely conditioned by land use, for example, forests have the largest and also those with the most recalcitrant material, compared to grass land and cropland characterized by the smallest one and with more labile form of C (Smith, 2007). Therefore land use, joined climate, is the main cause of variation in time of the

fluxes described above. In particular the main depletion in SOC pool occurs after deforestation, biomass burning and soil erosion (Fargione et al., 2008), moreover the indiscriminate and inappropriate land use, management practices (such as tillage, residues removal, excessive use of fertilizers and pesticides) and cultivation of peatlands even determine an increase in gaseous emissions to the atmosphere (Searchinger et al., 2008). The meta-analysis conducted by Guo & Gifford (2002) reported a decline of soil C stock after land use change from pasture to plantation (-10%), native forest to plantation (-13%), native forest to crop (-42%) and pasture to crop (-59%); while it showed a soil C stock increase after the opposite land use change, such as from native forest to pasture (+8%), crop to pasture (+19%), crop to plantation (+18%) and crop to secondary forest (+53%).

Therefore, agricultural sector is generally considered a net carbon source and the European largest biospheric C source with annual loss estimated between 41-115 MtC to 300 MtC (Janssens et al., 2003; Vleeshouwers & Verhagen, 2002) but the data are very uncertain. In an agroecosystem the C inputs, that exist only during a crop growing, are determined by NPP and the fraction of its remaining on the field, while the losses of C occur by decomposition and erosion (Freibauer et al., 2004). Instead in tree crops, widely diffused in the Mediterranean region, the C fluxes are under investigated, and few information are available about their C sink and their potential contribution to C sequestration (IPCC, 2007; Carlisle et al., 2006). In these particular agroecosystems, the C inputs are higher and the soil disturbance is lower than annual and herbaceous crops, but some data available only report the accumulation in the woody structures of peach, olive and apple tree crops after a few years (of 5 to 15 t of C ha^{-1}) (Butler & Landsberg, 1981; Rufat & DeJong, 2001; Sofo et al., 2005). It appears the importance of the preservation and increase of C pools, through appropriate land management practices, being essential to enhance also the quality of soil, water and other natural resources (Lal, 2011). The management practices can be divided in measures for reducing soil disturbance to prevent the C

losses such as reduced or zero tillage, set aside, growth of perennial crops, and measures for increasing soil-carbon inputs such as use of organic amendments (animal manure, sewage sludge, compost), improved rotations and irrigation, organic farming, conversion from arable agriculture to land use with higher C inputs or reduced disturbance (like bioenergy crop production, grassland, woodland) or to natural regeneration (Freibauer et al., 2004; Smith, 2004). It is necessary to underline that the soil C sink resulting from sequestration activities are not permanent and will continue only for the duration time of the C-sequestering management practice, in fact if a land management or land use is reversed, a loss of the C accumulated in soil occurs rapidly as its accumulation (Smith et al., 1996).

Therefore this new carbon sink, once established, must be preserved in perpetuity. Moreover the rate at which the C is removed from the atmosphere (sink strength) in soil becomes smaller as time goes on, as the soil carbon stock approaches a new equilibrium. At equilibrium, the sink has saturated: the carbon stock may have increased, but the sink strength has decreased to zero. The time to occur sink saturation (i.e. new equilibrium) is highly variable (in a temperate location, such as Europe, was estimated around 100 years). However, despite the limited duration of some C sequestration practices in cropland, they may have in short term a very important part of climate mitigation potential, over the environmental and economic benefits derived from a sustainable policy. In fact through the increase of organic C, improvement of soil structure, water holding capacity, fertility and resilience occur, thus soil quality, health and sustainability are in general positively affected.

Land-use practices like forests and agroecosystems management are the most promising fields for successful mitigation strategies in Kyoto Protocol framework.

1.1.3. Kyoto Protocol and soil C stock preservation

The Kyoto Protocol is an international agreement linked to United Nations Framework Convention on Climate Change (UNFCCC), which commits its parties by setting international binding emission reduction targets. It was adopted in Kyoto,

Japan, on 1 December 1997 and entered into force on 16 February 2005. The Kyoto Protocol recognizes the developed countries as the principal responsible for the current high GHGs emission in the atmosphere (result of the industrial activity) and places for them heavier burden in emission reduction. In Marrakesh, Morocco, in 2001, the detailed rules for the implementation of the Protocol were adopted and it was established the first commitment period: 2008-2012, at the end of this first period, in Doha, Qatar, on 8 December 2012, the second commitment period (2013-2020) was defined and the "Doha Amendment to the Kyoto Protocol" was adopted. As reported in Annex B of Kyoto Protocol, during the first commitment period, the 37 of industrialized countries that ratified the UNFCCC and the European Community, committed to reduce GHG emissions to an average of 5% against 1990 levels (only European Union committed to a reduction in CO_2 emissions to 92% of baseline), while, during the second one the Parties committed to reduce GHG emissions by at least 18% below 1990 levels; however, the composition of Parties in the second commitment period was different from the first (Kimble et al., 1998). The Kyoto Protocol allows C emissions to be offset by demonstrable removal of C from atmosphere, including land use and land management change as practices able to reduce atmospheric CO_2 levels, in fact, in Article 3.3 and 3.4 activities like afforestation, reforestation and improved management of agricultural soils (Smith, 2004) are mentionated. In particular, in Article 3.4, biospheric carbon sink and sources are included in attempts to meet limitation and reduction in C emission, including activities like re-vegetation, forest, cropland and grazing management. During the 17th Conference of Parties, in Durban 2011, these aspects were marked because, the forest management becomes mandatory rather than voluntary for all industrialized countries, and this, joined to the confirmation of the second commitment period of Kyoto Protocol and the possibility to include cropland management in carbon credits accountability (an increase in carbon stocks during the reference period will result in credits, while a decrease will result in debt), defined a

turning point in future of agriculture and forestry as a mitigation tool in fighting climate change.

1.1.4. *Carbotrees* project

Tree crops are account for more than 3 Mha of the Italian territory, of these, 0.65 Mha are vineyards and 1.1 Mha are olive groves, that represent the most important agricultural tree crops in Italy and throughout the Mediterranean area where it covers over 7 Mha. As reported above, because their higher C inputs and lower soil disturbance than annual and herbaceous crops, they could represent in the Mediterranean region the most promising sectors for national policies of climate change mitigation. The interest to provide a future scenario of the potential role of Italian forest and tree crops to meet the C emission reduction, represented the main purpose that drives the birth of Italian Carbotree project. The *CARBOTREES project* aims to improve the scientific knowledge on the potential for C sequestration and mitigation of GHGs emissions of Italian forest and tree crop systems. The project focused on different aspects within the forestry and tree crop sectors, such as: management forests and silvicultural options to increase the potential of carbon sequestration in existing forests; the potential carbon removals (in biomass and in soil) for areas subject to natural recolonization; potential C sequestration of different tree species used for reforestation; creation of scenarios of GHG emissions from forest fires at national scale, taking into account the changing climate and the application of methods to reduce fire risks; estimates of C sequestration potential of Italian tree crops (olive trees and vineyards); mitigation potential of tree residues used to produce bio energy/compost and evaluation of the life cycle of most representative tree crops (olive trees and vineyards).

1.2. Across the topics

Given the importance of soil C storage and its agronomic and environmental implication also in climate protection framework, the preservation and implementation of soil organic C reservoir is necessary. Obviously the soil C storage is strongly affect by soil chemical-physical properties, land use, topography, geographic allocation and climate condition. Therefore specific study are necessary to understand how and how much the boundary conditions can affect the soil C stock, allowing also to undertake appropriate management practices. Simplifying, the total C stored in soil has an amount and a residence time that depends on: 1) C pools (active/labile vs passive/recalcitrant) and their recycling; 2) main stabilization mechanism (physical/chemical) and 3) physical location (inter/intra aggregates vs free) (Six et al., 2000). This imply that each soil follows a different behaviour and could be affected differently by environmental conditions.

The three topic of this PhD thesis focused about three different aspects about C storage and land management practices, in particular the specific aims were:

a) to assess the potential improvement in C sequestration in tree crop systems compared to cropland, to understand if in Italian contest they could be a valid strategy in climate change mitigation;

b) to estimate the effects on soil C stock and soil quality of the agronomic use of olive pomace as soil amendment, in order to verify the sustainability of this practice;

c) to evaluate the organic C stability and distribution in size aggregates in soil developed in particular geographic and pedogenic conditions (as well as Andisols).

References

Albaladejo, J., Ortiz, R., Garcia-Franco, N., Navarro, A.R., Almagro, M., Pintado, J.G., Martínez-Mena, M. 2013. Land use and climate change impacts on soil organic carbon stocks in semi-arid Spain. *Journal of Soils and Sediments*, **13**(2), 265-277.

Butler, D.R., Landsberg, J.J. 1981. Respiration rates of apple trees, estimated by CO_2-efflux measurements. *Plant, Cell & Environment*, **4**(2), 153-159.

Carlisle, E.A., Steenwerth, K.L., Smart, D.R. 2006. Effects of land use on soil respiration: conversion of oak woodlands to vineyards. *Journal of Environtal Quality*, **35**(4), 1396-404.

Fargione, J., Hill, J., Tilman, D., Polasky, S., Hawthorne, P. 2008. Land Clearing and the Biofuel Carbon Debt. *Science*, **319**(5867), 1235-1238.

Freibauer, A., Rounsevell, M.D.A., Smith, P., Verhagen, J. 2004. Carbon sequestration in the agricultural soils of Europe. *Geoderma*, **122**(1), 1-23.

Guo, L.B., Gifford, R.M. 2002. Soil carbon stocks and land use change: a meta analysis. *Global Change Biology*, **8**(4), 345-360.

IPCC 2007. IPCC Climate Change: The Physical Science Basis, Solomon, S., et al. (eds), Cambridge University Press, Cambridge.

Janssens, I.A., Freibauer, A., Ciais, P., Smith, P., Nabuurs, G.-J., Folberth, G., Schlamadinger, B., Hutjes, R.W.A., Ceulemans, R., Schulze, E.D., Valentini, R., Dolman, A.J. 2003. Europe's Terrestrial Biosphere Absorbs 7 to 12% of European Anthropogenic CO_2 Emissions. *Science*, **300**(5625), 1538-1542.

Jobbágy, E.G., Jackson, R.B. 2000. The vertical distribution of soil organic carbon and its relation to climate and vegetation. *Ecological Applications*, **10**(2), 423-436.

Kimble, J.M., Follett, R.F., Cole, C.V. 1998. The Potential of U.S. Cropland to Sequester Carbon and Mitigate the Greenhouse Effect. Taylor & Francis.

Lal, R. 2011. Sequestering carbon in soils of agro-ecosystems. *Food Policy*, **36,** Supplement 1, S33-S39.

Le Queré, C, 2008. Global Carbon Budget. University of East Anglia, UK.

Lloyd, J., Farquhar, G. 1996. The CO_2 dependence of photosynthesis, plant growth responses to elevated atmospheric CO_2 concentrations and their interaction with soil nutrient status. General principles and forest ecosystems. *Functional Ecology*, **10**(1), 4-32.

Prentice, I.C., Farquhar, G., Fasham, M., Goulden, M., Heimann, M., Jaramillo, V., Kheshgi, H., LeQuéré, C., Scholes, R., Wallace, D.W. 2001. The carbon cycle and atmospheric carbon dioxide.

Rufat, J., DeJong, T.M. 2001. Estimating seasonal nitrogen dynamics in peach trees in response to nitrogen availability. *Tree Physiology*, **21**(15), 1133-1140.

Schulze, E., Heimann, M. 1998. Carbon and water exchange of terrestrial systems. *Asian change in the context of global change*, **3**, 145-161.

Searchinger, T., Heimlich, R., Houghton, R.A., Dong, F., Elobeid, A., Fabiosa, J., Tokgoz, S., Hayes, D., Yu, T.H. 2008. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emissions from Land-Use Change. *Science*, **319**(5867), 1238-1240.

Six, J., Elliott, E., Paustian, K. 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. *Soil Biology and Biochemistry*, **32**(14), 2099-2103.

Smith, P. 2004. Carbon sequestration in croplands: the potential in Europe and the global context. *European Journal of Agronomy*, **20**(3), 229-236.

Smith, P. 2007. Land use change and soil organic carbon dynamics. *Nutrient Cycling in Agroecosystems*, **81**(2), 169-178.

Smith, P., Powlson, D.S., Glendining, M.J. 1996. Establishing a European soil organic matter network (SOMNET). In: Powlson, D.S., Smith, P., Smith, J.U.

(eds) Evaluation of soil organic matter models using existing, long-term datasets, NATO ASI Series I, vol. 38. Springer-Verlag, Berlin, pp 81–98

Sofo, A., Nuzzo, V., Palese, A.M., Xiloyannis, C., Celano, G., Zukowskyj, P., Dichio, B. 2005. Net CO2 storage in mediterranean olive and peach orchards. *Scientia Horticulturae*, **107**(1), 17-24.

Stockmann, U., Adams, M.A., Crawford, J.W., Field, D.J., Henakaarchchi, N., Jenkins, M., Minasny, B., McBratney, A.B., Courcelles, V.d.R.d., Singh, K., Wheeler, I., Abbott, L., Angers, D.A., Baldock, J., Bird, M., Brookes, P.C., Chenu, C., Jastrow, J.D., Lal, R., Lehmann, J., O'Donnell, A.G., Parton, W.J., Whitehead, D., Zimmermann, M. 2013. The knowns, known unknowns and unknowns of sequestration of soil organic carbon. *Agriculture, Ecosystems & Environment*, **164**, 80-99.

Vleeshouwers, L.M., Verhagen, A. 2002. Carbon emission and sequestration by agricultural land use: a model study for Europe. *Global Change Biology*, **8**(6), 519-530.

Waring, R.H., Landsberg, J.J., Williams, M. 1998. Net primary production of forests: a constant fraction of gross primary production? *Tree Physiology*, **18**(2), 129-134.

Chapter 2- SOM: composition, stability, dynamic and study approaches

2.1. The Soil Organic Matter

Organic matter in soil plays an important role in determining soil water-holding capacity and soil structure, it provides a long-term store of nutrients needed by plants (Trumbore, 1997). The partial decomposition of plant debris by microbial activity is the main process in soil organic matter formation (Paul, 2014). The resulting material as proposed firstly by Piccolo (2002), and now accepted in literature, is a supramolecular association of self-assembling heterogeneous and relatively small molecules, deriving from the degradation and decomposition of dead biological material, stabilized predominantly by weak dispersive forces instead of covalent linkages, such as hydrophobic (van der Waals, π-π and CH-π) and hydrogen bonds; the results are apparent large molecular size of humic substance. Therefore in this view it is overcome the traditional assumption that consider humic substances as complex polymers in soil. It was proposed recently the theory of humus formation by transformation and condensation processes of plant structural compounds, following the hypothesis of *de novo* formation of humic polymers through the microbial synthesis and stabilization by the soil matrix (Schmidt et al., 2011) (Figure 2.1). Simplifying it is possible to affirm that humic substances are a complex mixture of microbial and plant polymers and their degradation products are associated in super structures stabilized by hydrogen and hydrophobic bonds (Cotrufo et al., 2013). In fact, Schmidt et al. (2011) highlighted that humic substances, traditionally considered the most stable soil organic matter (SOM) fractions composed by large and complex macromolecules, represent only a small part of total organic matter, compared to smaller and simpler molecular structures find in soil by direct in situ observation (Kleber & Johnson, 2010; Lehmann et al., 2008; Lützow et al., 2006; Sutton & Sposito, 2005). Moreover the molecular structures of plant input and organic matter

have a secondary role in determining carbon stability and high residence time, while more important are the biotic and abiotic environmental conditions. Therefore the persistence of organic matter in soil is due to complex interactions between the organic matter and environment, involving compound chemistry, reactive mineral surface, climate, water availability, soil acidity, soil redox state (Figure 2.1) (Schmidt et al., 2011).

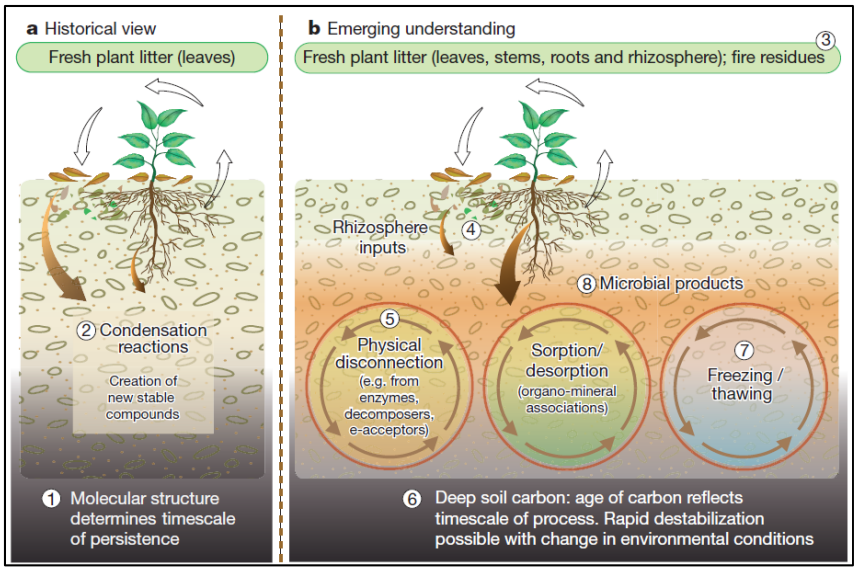


Figure 2.1. Schematic comparison between historical (a) and emerging views (b) of soil C cycling. (Schmidt et al., 2011).

As reported above, microbial products are the largest contributor to stable SOM, and their accumulation is determined by the interaction with soil matrix (Knicker, 2011). Cotrufo et al. (2013) proposed the substrate use efficiency theory to explain the organic matter accumulation in soil: the amount of soil microbial activity products is function of the substrate use efficiency (SUE), namely the portion of assimilated substrate used for growth and enzyme production respect to the portion respired or mineralized, and it depends by substrate quality and degradative efficiency of microbes. Higher value of SUE are reported for simple metabolic compounds (Dijkstra et al., 2011), compared with complex structural plant components (Bahri et

al., 2008), because the protected polymers must first be depolymerized by extracellular enzymes prior to assimilate with a consequent reduction in SUE. The permanence in soil of microbial by-products is determined by complex physical (due to a spatial inaccessibility for microbes) and chemical (due to its functional group composition and molecular structure) mechanism of interaction with soil matrix that protect the SOM from decomposition (Six et al., 2006). The integration of chemical and physical characteristics of both SOM and soil minerals, produces a matrix stabilization process (Cotrufo et al., 2013), controlled by the quality and amount of silt and clay in soil (Lützow et al., 2006; Sollins et al., 1996). Briefly, the main kind of interaction of soil organic matter are: in acid soil interaction with Fe-, Al-, Mn-oxides, as clay coating, concerning large superficial area and also the formation of micropores and microaggregates; in neutral and calcareous soil bound between largely negatively charged SOM and negatively charged phyllosilicate by a polyvalent cations (as Ca^{2+}) as bridge; in soils, like Andisols, rich in short order silicates (as allophane) strong organo-mineral interactions (Buurman et al., 2007; Dümig et al., 2012; Mikutta & Kaiser, 2011).

Over the microbial products, also plants compounds could be stabilized. In fact, the roots exudates, as well as organic acids (oxalate, malate, citrate) generally considered labile compounds mineralizable in few hours following the release by roots (Rasse et al., 2005), are characterized by a negative charge that cause their sorption on mineral phase by cation bounding (Jones & Edwards, 1998) (Figure 2.2). For example, Fe-oxides, that possess most of the available surface area mineral in soils, appears effective sorbants of soluble organic matter (Kaiser & Zech, 1998), playing an important role in organic compound stabilization (Saggar et al., 1996; Torn et al., 1997).

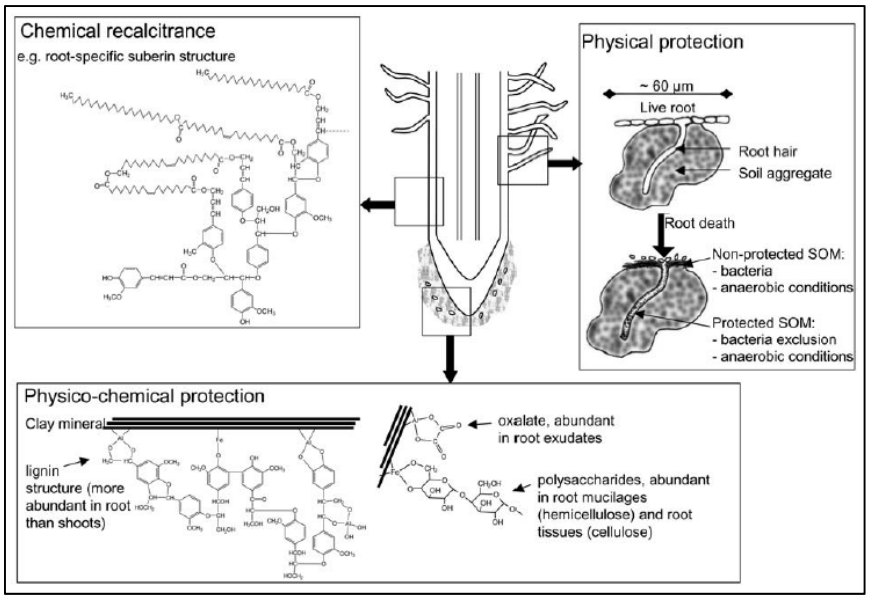


Figure 2.2 Representation of the three main processes resulting in the specific protection of root C in soils (Rasse et al., 2005).

Each mechanism influences SOM stabilization differently over time: the chemical properties and physical inaccessibility operate over short to medium term (decades), whereas matrix stabilization over the long term (centuries) (Kögel-Knabner et al., 2008). It is necessary to highlight that an important role in plant litter decomposition and decay is represented by its quality, in general a faster decay occurs with low concentration of chemically recalcitrant substrates in litter (Zhang et al., 2008). The C chemistry (concentration of water solubles, celluloses, lipids, lignin) and N content are the two main parameters defining litter quality, that determine litter decomposability: commonly "high-quality" litter decompose faster than "low-quality" litter. Therefore SOM would be formed by high quality litter than to low quality one, and accumulate in long term, after a soil matrix stabilization.

2.2. SOM fractions

The soil organic matter (SOM) fractions were classified by Strosser (2010) in: labile, stable and inert. The **Labile** SOM is defined as a quickly reactive organic matter, important source of nutrients soil microbial community, with an half-life ranging

from days to few years and for this its provides a short-term organic matter turnover during the year. The **Stable** SOM is considered a reservoir of less decomposable organic matter, for its organic matter chemistry and composition, this pool is also involved in soil aggregates formation, and its half-life is between years and decades. The **Inert** SOM is defined as the non-reactive organic matter SOM fraction, probably due to a physico-chemical protection against decomposition, affecting the physical properties of the soil, and its half-life is between decades and centuries (Strosser, 2010). However also other SOM classification are available in literature, for example Trumbore (1997) reported that soil is composed by three C pools: "**active**" pool (root exudates, microbial cell contents and rapidly decomposable components of fresh plant litter), "**passive**" pool (highly stabilized organic material, typically associated with mineral surface or very stable aggregates) and "**intermediate**" pool (C pool not clearly defined, consisting in structural components of plants more resistant to decay, or organic compounds that have been stabilized by their association with soil minerals or aggregates structures) (Figure 2.3). These SOM pools derive from a decomposition rates that cluster at three very different time scales: sub-annul, decadal-century and longer (Torn et al., 2009; Trumbore, 1997).

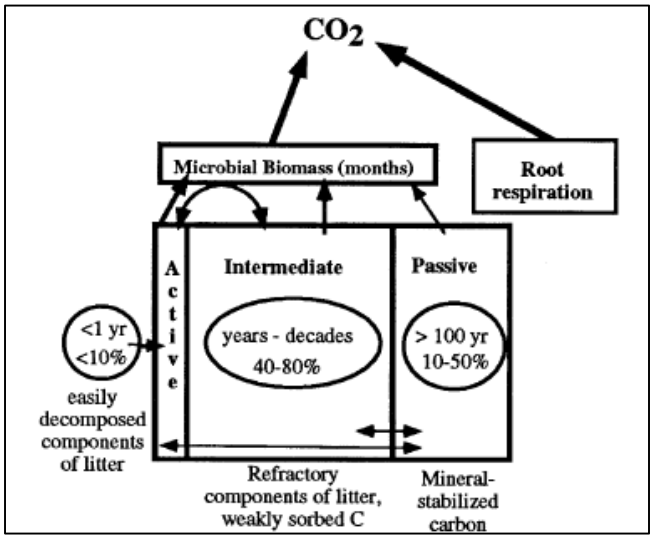


Figure 2.3 Model of SOM dynamics proposed by Trumbore (1997).

The identification and characterization of SOM fraction, anyway, is function and strictly related to the methodological approach used for their separation and to the study objective. There are numerous approaches to separating SOM pools for analysis, each one characterized by specific base concepts and provide various kind of information. Usually the common objective of fractionation is to reduce the variation of chemical and physical properties and the C-cycling time among fractions and compared to bulk soil, nevertheless the SOM separated still remain a mixture of heterogeneous compounds (Torn et al., 2009).

2.3. Methods to separate and study SOM fractions

The physical fractionation methods are based on the premise that the association of soil particles and their spatial arrangement play a key role in SOM dynamics, because bioaccessibility is a prerequisite for decomposition. Consequently, the OM turnover, is regulated by biological processes, under the overall regulation of soil structure and substrate availability for decomposers, that depends both on substrate chemical nature and also soil mineral association, like the formation of organo-mineral complex (Christensen, 2001; von Lützow et al., 2007). Physical fractionation of soil according to the size and density of particles is achieved by applying various degrees of dispersion to break bonds between the elements of soil structure, and it allows the separation of both uncomplexed OM and variously sized organo-mineral complexes (Christensen, 2001).

Downstairs, three different physical fractionation methods will be briefly described.

2.3.1. Aggregates fractionation

The goal of **aggregates fractionation** is to isolate C pools in different soil physical structures; it is based on the assumption that soil structure is a major control on SOM turnover through physical protection, and the soil aggregates are considered the ecologically meaningful fraction (Moni et al., 2012). Aggregates fractionation (Figure 2.4) allow the separation of free SOM and protected SOM, as well as SOM

occluded in secondary organo-mineral assemblages of different size (von Lützow et al., 2007); the isolation of active from intermediate and passive SOM pools is performed by the separation of free SOM (active pool) from occluded SOM in macro and microaggregates (intermediate pool) and SOM in clay microstructures (passive pool) (von Lützow et al., 2007). An example of aggregate fractionation is those proposed by Six et al. (2000), that by dry or wet sieving and slaking, SOM is divided into relatively homogeneous and different functional fractions. The **unprotected organic matter**, composed by plant residues, partial decomposed microbial by-products, but also seed and microbial debris (Besnard et al., 1996; Oades et al., 1987) not closely associated with soil minerals, is measurable either as Light fraction (LF) or POM fraction, that despite some differences in their characteristics, could be considered similar conceptual pools; both LF and POM, especially coarse POM (>250 μm) are relatively easily decomposable (Six et al., 1999). The POM is isolated by size separation: unprotected POM is defined as the 53-200 μm size POM not contained within microaggregates and protected POM as 53-200 μm sized fine POM occluded within microaggregates. Other three pools are identified by Six et al. (2000) and are denominate as protected SOM pools, according to the three stabilization mechanisms considered by the authors (chemical stabilization, physical protection and biochemical stabilization): **microaggregate-protected SOM** (53-250 μm), **silt and clay-protected SOM** (organo-mineral complex <53 μm), that together represents part of the slow pool (or intermediate pool), and **non-hydrolyzable fraction of the silt and clay-associated C** that represents the biochemically protected pool, that is comparable to the passive pool.

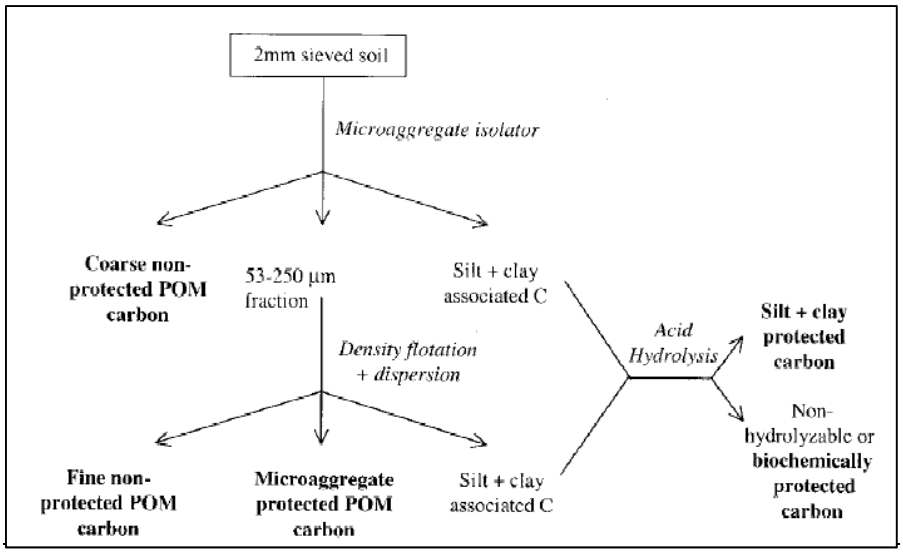


Figure 2.4 Conceptual model of aggregates fractionation scheme (Six et al., 2002).

2.3.2. Particle size fractionation

Particle size fractionation is based on the concept that SOM associated with soil particles characterised by different size and mineralogical composition, has different structure and function (Christensen, 1992). In this perspective, quartz particle that dominate the sand fraction exhibit only weak bonding affinities to SOM, while clay-size particle provide a large surface area and numerous reactive sites where SOM can be sorbed by strong ligand exchange and polyvalent cation bridges (Sposito et al., 1999). Assuming sorption as important stabilization mechanism, SOM within the sand fraction is identified as active pool, while SOM in silt and clay fractions as intermediate and passive pool (von Lützow et al., 2007). However contrasting results in literature highlight that this kind of fractionation, provides not homogenous fractions in term of turnover time.

2.3.3. Density fractionation

The **density fractionation** exploits the differences in density between particulate OM and mineral associated OM. On the base of this assumption, density fractionation is applied to isolate light fraction (LF), that is not associated with soil minerals, consisting principally in partially decomposed plant residues that floats in a dense

liquid (1,6-2 g cm^{-3}), from the denser heavy fraction (>1,6-2 g cm^{-3}), the organo-mirneral complexes, that precipitates in the dense solution (Torn et al., 2009). Density fractionation is performed by an aqueous solutions of inorganic salt, like sodium polytungstate ($Na_6(H_2W_{12}O_{40})$, SPT). The OM stability increases from LF to HF, consequently a slower turnover rate is recoded for occluded OM compared to the free one. If density fractionation is applied with the intention to achieve active, intermediate and passive pools, like aggregate and particulate fractionation methods, also in this case the result is a rough differentiation of active and passive OM. In fact, while LF is a good representation of the active pool, the HF is too heterogeneous and does not represent functional pools, because contributes either to passive and intermediate pool. In fact the physical fractionation captures the effects of the spatial arrangement of primary and secondary organo-mineral particles on SOM dynamics, without consider the chemical agents for SOM stabilization (Olk & Gregorich, 2006). However, if it is performed as pre-treatment for further functional fraction differentiation, the density fractionation represents an useful tool (von Lützow et al., 2007). For this reason physical and chemical methods could be combined, in order to exploit chemical characterization derived from the application of chemical fractionation, to elucidate molecular-level interactions between SOM and mineral soil particle (Olk & Gregorich, 2006). In general chemical fractionation methods are based on the hydrolysability of SOM with water or acids and the resistance of SOM to oxidation, these could be applied not only on bulk soil sample but also on fractions from physical fractionation procedure. For example Trumbore & Zheng (1996) proposed the combination of firstly density separation, allowing the removal of LF from the sample, and a further base extraction and acid hydrolysis on HF allowing a chemical characterization of the SOM more related to pedogenesis (Kögel-Knabner, 2000). The whole procedure is designed to separate a mineral-free LF, a chemically labile mineral-associated OM fraction (called hydrolysable) and a chemically recalcitrant mineral-associated OM (non-hydrolysable) that is the residual fraction

collect at the end of the procedure (Castanha et al., 2008), namely pools that turnover on different time scale and identifiable as active, intermediate and passive pool respectively. In particular the chemical fractionation on HF proposed by Trumbore & Zheng (1996) uses basic and acid hydrolysis. The basic hydrolysis is performed by a mixture of 0.1 M NaOH+0.1M $Na_4P_2O_7$ that cause the extraction of mineral associated organic matter. In fact the Na^+ from NaOH replaces H^+-bridges within SOM, causing SOM solubilization and also rearrangement of organic association (Piccolo, 2002), while polyvalent cation bridges between SOM and soil minerals are disrupted by extraction with $Na_4P_2O_7$: in this way the fraction of SOM bound to clay minerals by metal bridges and SOM complexed by polyvalent cations are hydrolysed. Moreover the Na^+ ions also interferes with the flocculation of clays causing a disaggregation. Acid hydrolysis with 6M HCl, removes carbohydrate and protein materials by disruption of hydrolytic bonding, leaving the more biologically recalcitrant long-chain alkyls and aryl materials, waxes, lignin, and other aromatics molecules (Paul et al., 2006). Polyvalent cations involved in clay flocculation and microaggregation are also removed by acid treatments, because it dissolves sesquioxide minerals and remove labile SOM complexed with Fe and Al, rendering occluded and complexed SOM soluble (Oades, 1988). Acid hydrolysis shows an unspecific reactivity and mobilises SOM stabilised by different mechanisms, such as occluded SOM, SOM sorbed by polyvalent cation bridges and complexed SOM. However, the residual SOM fraction is still heterogeneous and its stabilization mechanisms are not well understood (Trumbore & Zheng, 1996).

2.4. ^{14}C: tool to study SOM fractions stability and dynamic

Radiocarbon (^{14}C) is a useful tool for studying C exchange between terrestrial ecosystems and atmosphere (Trumbore, 2009). It is a cosmogenic radionuclide, which means it is constantly created by the interaction of cosmogenic rays with the atmosphere and Earth's surface, due to the interaction between cosmogenic rays and atmospheric nitrogen, ^{14}N (Marzaioli, 2011):

$$^{14}N+n= {}^{14}C+p$$

The highest atmospheric production rate is restricted within to 15-18 km altitude belt (lower stratosphere), because of the higher thermal neutron concentration. A higher ^{14}C production occurs at the pole compared to equator due to the earth magnetic field. Radiocarbon is fast transferred to the troposphere, mainly in the late winter/early summer period at the meld latitudes driven by the down welling currents of the climatic cells, and oxidized to $^{14}CO_2$ allowing its entry in global carbon cycle (Marzaioli, 2011). The ^{14}C nucleus is unstable and will spontaneously emit a β particle (electron), decaying to ^{14}N with a half-life of 5730 years:

$$^{14}C = {}^{14}N+ \beta^{-}+\nu$$

where β^- is the electron and ν the antineutrino produced.

Because of the troposphere receives an input flux of ^{14}C from the stratosphere and loses ^{14}C in a output fluxes corresponding to radioactive decay and to transfer to biosphere and oceans, it constitutes the steady state reservoir of ^{14}C. In this perspective, the possible variation, related to change of solar activity and anthropogenic impact are considered neglecting. Radiocarbon content of living organism is approximately in dynamic equilibrium with the radiocarbon content of the atmosphere. The decay of organic and/or inorganic radiocarbon stats after death

of living material, or at the end of exchanging flux with atmosphere, accords to radioactive exponential decay law:

$$\frac{dN}{dt} = -\lambda N \quad ; \quad N(t) = N_0 e^{-\lambda t}$$

where *N(t)* is the quantity at time t, *N0=N(0)* is the initial quantity, λ is decay constant (for radiocarbon 8267 y^{-1}), *t* is time spent since death occurred (radiocarbon age). When the C atoms are removed from contacts with the atmosphere and stored with no further exchange with other reservoirs, the reduction of the $^{14}C/^{12}C$ ratio, caused by the radioactive decay, compared with the original atmospheric CO_2 from which it was derived, indicates the time elapsed since removal (Trumbore, 2009). In fact, in some C compartment, such as C pool like soil organic matter that receives constantly new C input from plant and loses C for example through decomposition, the $^{14}C/^{12}C$ ratio provides information about the rate of decomposition and radioactive decay (Torn et al., 2009; Trumbore, 2000). However radiocarbon has also anthropogenic origins, and human activities represent an additional source directly accessible for biosphere (Marzaioli, 2011); in fact the thermonuclear weapon test during the early 1960s, produced an increase, about a doubling, in $^{14}C/^{12}C$ ratio in Northern Hemisphere atmospheric CO_2 (Figure 2.5). Therefore this bomb-produced ^{14}C serves as global tracer for C exchange among the atmosphere, ocean and terrestrial C reservoirs for decades from the beginning of the inputs. Since the 1964 the amount of ^{14}C in atmospheric CO_2 has decreased, initially because the blending of the atmosphere of Northern and Southern Hemisphere caused a dilution of bomb ^{14}C, than over subsequent decades as bomb ^{14}C was incorporated into ocean and terrestrial C pools (Naegler & Levin, 2006; Randerson et al., 2002) .

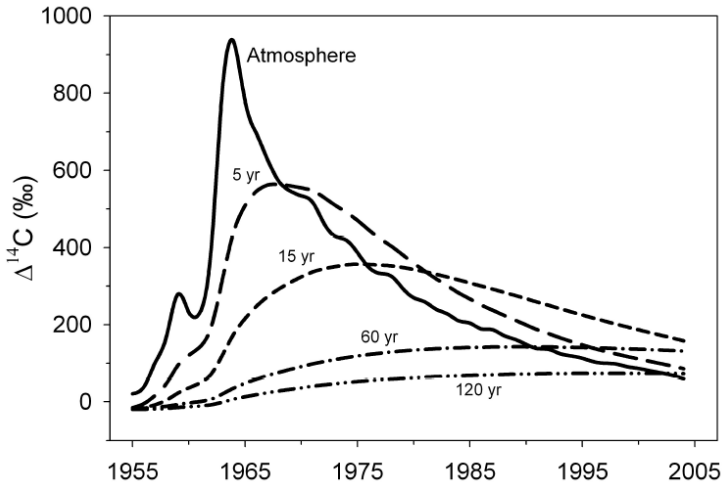


Figure 2.5 Change in atmospheric ^{14}C (expressed as per mil variation in $^{14}C/^{12}C$ ratio relative to a standard) with time in the northern hemisphere (heavy solid line) since 1955. The lighter lines show the evolution of ^{14}C for homogeneous and steady-state reservoirs with different turnover times: 5, 15, 60, and 120 years (Torn et al., 2009).

The radioactive decay of ^{14}C is comparatively small on this timescales, and the current decrease in the atmospheric $^{14}CO_2$ signature from one year to next is greater than the precision of the ^{14}C measurement. Since 1963, the record of radiocarbon allows us to infer the timescales for C exchange with the atmosphere in a given reservoir on annual to decadal timescales. The radiocarbon signature of atmospheric CO_2 in the 80's and 90's declines at annual rate of 8‰ y^{-1}(Levin et al., 1992) and between 1996 to present of about 4‰ y^{-1} (Levin & Hesshaimer, 2000). Because of the annual decreasing rate of the bomb radiocarbon curve is higher than the mean precision in the $\Delta^{14}C$ measurements, it is possible, in the last 40 years, dating organic materials with an error less than 1 year. Thus, the $^{14}CO_2$ fixed during the photosynthesis in organic compounds, enters in soil by litter or roots exudates, and in this way became a tracer of soil organic C. Therefore the bomb radiocarbon is an important tool to study soil organic matter dynamic, useful to determine SOM age and stability, even if on this topic, ^{14}C still is underutilized (Trumbore, 2009).

2.4.1. SOM turnover and percent in modern carbon

The SOM is a dynamic entity, because of SOM is in a continuum state of flux within the pools and SOM stock can vary in response to environmental factors (climate, vegetation, land use, management) or rate of mineralization; for these reasons the turnover of SOM is probably more significant than the size of SOM stock (Six & Jastrow, 2002). The turnover (τ) of an element in a pool is determined by the balance between input (I) and output (O) through the pool, and represent the time for a reservoir to completely empty if there were no further inputs. For a soil C reservoir at steady state (I=O), the τ is calculated dividing the mass of SOM (C) by the total flux (S) from the reservoir:

$$\tau = \frac{C}{S}$$

The turnover time is quantified as the element's mean residence time (MRT) and it is defined as the average time spent in the reservoir or pool at the steady state by the individual C atoms, or the average time required to completely renew the content of the pool (Six & Jastrow, 2002; Torn et al., 2009). The dynamic of SOM is described by the first-order model, which assume constant zero-order input with constant proportional mass loss per unit time (Jenny, 1980):

$$\frac{\partial S}{\partial t} = I - kS$$

where *S* is SOM stock; *t* is the time, *k* is the decomposition rate and *kS* is equivalent to output (O). At the equilibrium (I=O), the MRT can be calculated by:

$$MRT = \frac{1}{k}$$

The unknown *k* is calculated as: $k = \frac{I}{S}$, assuming a steady state ($\frac{\partial S}{\partial t} = 0$).

The half-life of SOM ($T_{1/2}$) is defined as the time required for half of the currently existing stock to decompose and it could be calculated by:

$$MRT = \frac{T_{1/2}}{\ln 2}$$

The presence of ^{14}C with an half-life of 5570 years in plants and the transformation of this ^{14}C into SOM with little isotopic discrimination, allows the SOM to be dated, providing an estimate of the age of the SOM (Six et al., 2002). Thermonuclear bomb test that during 1950s and 1960s caused a significant increase in the atmosphere of ^{14}C and a drastic decrease when the test were halted, determine the incorporation of bomb-produced radiocarbon into SOM, allowing the estimate of the turnover of SOM (like an in situ trace experiment) (Six & Jastrow, 2002).

The ^{14}C samples abundance is determined by a directly measure of ^{14}C atoms and the $^{14}C/^{12}C$ ratio, by accelerator mass spectroscopy (AMS). This technic reports ^{14}C data as the ratio of ^{14}C activity in the sample to that of a known standard, as *delta* notation that is used to define the abundance of stable isotope with respect to given standard (Marzaioli, 2011):

$$\delta'X = \left(\frac{(X^r/X^a)_s - (X^r/X^a)_{std}}{(X^r/X^a)_{std}} \right) x\ 1000$$

where X^r is rare isotope abundance; X^a is abundant isotope abundance; *s* is sample; *std* is standard

In the specific case of ^{14}C, the standard is corrected to 0,95 time the activity of an oxalic acid standard (OX_1), which is normalized to $\delta^{13}C$ of -19‰ (Stuiver and Polach, 1977). The sample is also normalized for ^{13}C content, the activity of the sample (As) with a $\delta^{13}C$ of δ notation is corrected to a constant ^{13}C abundance (-25‰), using the following equation (Torn et al., 2009):

$$A_{SN} = A_S \frac{(1 - 25/1000)^2}{(1 + \delta/1000)^2}$$

where A_{SN} is the ^{13}C corrected sample activity and AS the un-^{13}C corrected activity of the sample.

The correction of radiocarbon data to a common $\delta^{13}C$ value is necessary to eliminate the effects of isotope fractionation, in fact the standard approach to correcting for ^{13}C is applicable when fractionation is due to mass-dependent processes, such as biological fixation of CO_2. The $\delta^{13}C$ difference between atmospheric CO_2 and C fixed during photosynthesis by C3 plants is approximately 20‰; assuming that the fractionation of ^{14}C is roughly twice that of $\delta^{13}C$, since the mass difference between 12 and 14 is twice that between 12 and 13, the difference in ^{14}C abundance, between atmospheric CO_2 and photosynthates will be approximately 40‰, even though both CO_2 and photosynthates are the same "age" (Torn et al., 2009).

For ^{14}C data is used the term "fraction modern" ($F^{14}C$) (Reimer et al., 2004):

$$F^{14}C = \frac{A_{SN}}{A_{ON}} = \frac{\left(\frac{^{14}C}{^{12}C + ^{13}C}\right)_{sample(-25)}}{0.95\left(\frac{^{14}C}{^{12}C + ^{13}C}\right)_{OX1(-19)}}$$

where A_{ON} is 0.95 times the measured activity of the OX_1 standard normalized to a $\delta^{13}C$ of -19‰, and A_{SN} the ^{13}C corrected sample activity reported above.

Each measured radiocarbon ratio, both A_{SN} and A_{ON}, has to be normalized to the atmospheric reference CO_2 radiocarbon content value accounting for mass dependent fractionation by means of its actual $\delta^{13}C$. As a standard is defined by means of a constant isotopic ratio, OX measured value needs to be corrected for radioactive decay (using 1950 as reference year) and multiplied for 0.95 to match the 1890 pre fossil fuel diluted ^{14}C atmosphere (Marzaioli, 2011).

The conventional radiocarbon age is calculated considering the decay law:

$$^{14}\text{C age} = -8033 \ln \text{F}^{14}\text{C} \quad \rightarrow \quad ^{14}\text{C age} = -8033 \ln(\text{A}_{\text{SN}}/\text{A}_{\text{ON}})$$

where 8033 y is the Libby mean life of radiocarbon, used as convention, while the true mean life is 8267 y.

Radiocarbon age is referred to 1950 and samples with more ^{14}C than the 1950 atmosphere are commonly reported as "modern". Because of the activity of OX_1 changes through time as ^{14}C in standard decay, for an open and dynamic system, such as the soil, it is necessary to refer to a standard with a constant value, for this reason Stuiver & Polach (1977), proposed an absolute international standard activity (A_{abs}) that incorporate a yearly correction for decay in OX_1 standard:

$$A_{abs} = A_{ON}\, e^{\lambda(y-1950)}$$

where y is the year of sample collection, λ is the true radiocarbon decay (λ =1/8267 y^{-1}). The ^{14}C units in a sample are reported as $\Delta^{14}C$, that is the deviation in parts of thousand (‰) for the absolute standard:

$$\Delta^{14}C = \left[\frac{A_{SN}}{A_{abs}} - 1\right] x1000 = \left[F^{14}C\, e^{\left(\frac{-(y-1950)}{8267}\right)} - 1\right] x1000.$$

Positive values of $\Delta^{14}C$ indicate the presence of bomb-produced ^{14}C, conversely, negative values indicate the predominance of C fixed from the atmosphere long enough ago, for a significant radioactive decay of ^{14}C to have occurred (Torn et al., 2009). The analytical precision typically reported with the data is the 1 sigma error, determined from counting statistics and propagating laboratory errors; typical precision reported for samples with $F^{14}C$ ~1 is ±0.005 (± 5‰ for $\Delta^{14}C$) and for high-precision analyses low as 0.001 (Marzaioli, 2011).

2.4.2. ^{14}C in SOM fractions

Radiocarbon is used as indicator of the rate of exchange of carbon between soil and atmospheric CO_2. In fact soil organic matter is a dynamic (the C is continuously lost and added) and heterogeneous reservoir with a variety of turnover time, in which the ^{14}C of SOM represents the average ^{14}C age of a C atom in the soil reservoir (Figure 2.6) (Six et al., 2002).

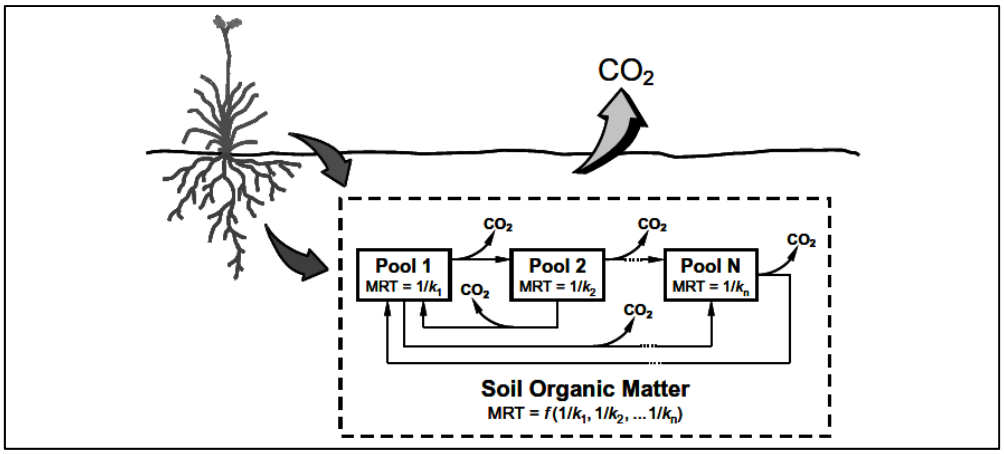


Figure 2.6. A schematic representation of turnover in SOM. The balance between soil inputs and outputs determine soil turnover time, and because of total SOM consists of different pools that are turning at different rates, the MRT of total SOM is function of the turnover rates of its constituent pools (Six, 2002).

However, it is possible derive the most meaningful results if ^{14}C-dating is applied on functional and homogeneous SOM pools, because the ^{14}C signature of bulk soil is governed by mineral associated organic matter (Schulze et al., 2009). The models that describe the dynamics of accumulation and turnover of organic carbon, recognize components of SOM that turnover on annual (active), decadal (intermediate) and centennial to millennial (passive) time scales (Trumbore & Zheng, 1996). The stable organic C pools in soil reside long time, so a significant decay of ^{14}C occurs, therefore slower is the turnover of a fraction, more ^{14}C-depleted it appears. Conversely, the fast-cycling pool, such as SOM "active pool", appears enriched in bomb^{14}C in comparison to bulk soil (Schulze et al., 2009; Torn et al., 2009). The OC in soil is also function of the decomposition process, that can be divided into many following phases. The first phase of decomposition shows a turnover time of about 1-2 years (for example in temperate clime) and a loss of about a quarter to two-thirds of the initial C (active or labile OM). The following phase with slow decomposition rates, with a total loss of about 90% OM (identifiable as intermediate pool) and lasting about 10-100 years. The complete decay process comprise a third phase with a very slow decomposition rates and long turnover times of about 100- >1000 years

(Lützow et al., 2006). This slow OM pool is responsible for the long-term stabilization of C in soil (Falloon & Smith, 2000). The stabilization mechanisms of soil organic matter play an important role in the determination of SOM turnover time over the chemical recalcitrance that also occurs (Castanha et al., 2008). Whereby in mineral-associated organic matter, as well as the dense fraction, a slow turnover rate is generally observed, but at the same time, itself includes components of very different ages. The scope to subject the heavy fraction (HF) to a chemical hydrolysis, is to obtain a residual organic matter depleted in ^{14}C compared to bulk soil, that generally matches the century-to-millennial cycling C, so-called "passive pool", because the chemical treatment solubilizes relatively more labile, more rapidly cycling carbon and ^{14}C-enriched components of the dense mineral associated OM, identifiable with "intermediate pool" (Figure 2.7) (Trumbore, 1993; Trumbore & Zheng, 1996). The employment of fractionation method that comprises either a physical and chemical fractionation is useful for a good isolation of the most stable OM; in fact during the basic and acid hydrolysis, the younger and potentially degradable compounds are removed, while the non-hydrolysable C fraction is left and is considered representative of recalcitrant OM. Therefore a short MRT of SOM fractions indicate low persistence which translates into high turnover rates if the system is at steady state (Trumbore, 2000). However Trumbore (2000) highlights two assumption to bring in consideration when ^{14}C is used to determine turnover times for SOM fractions. Firstly, the use of a single decomposition rate assumes that any given C atom in SOM fraction will decay at the same probability, but this is never true for OM in bulk soil and could be only an approximation for SOM fractions isolated using density or chemical separations; second, the C added to SOM fraction each year has the $\Delta^{14}C$ signature of that year's atmospheric CO_2, namely the C added is fresh photosynthate and no lag time exists between C fixation and addition to soil as dead organic matter.

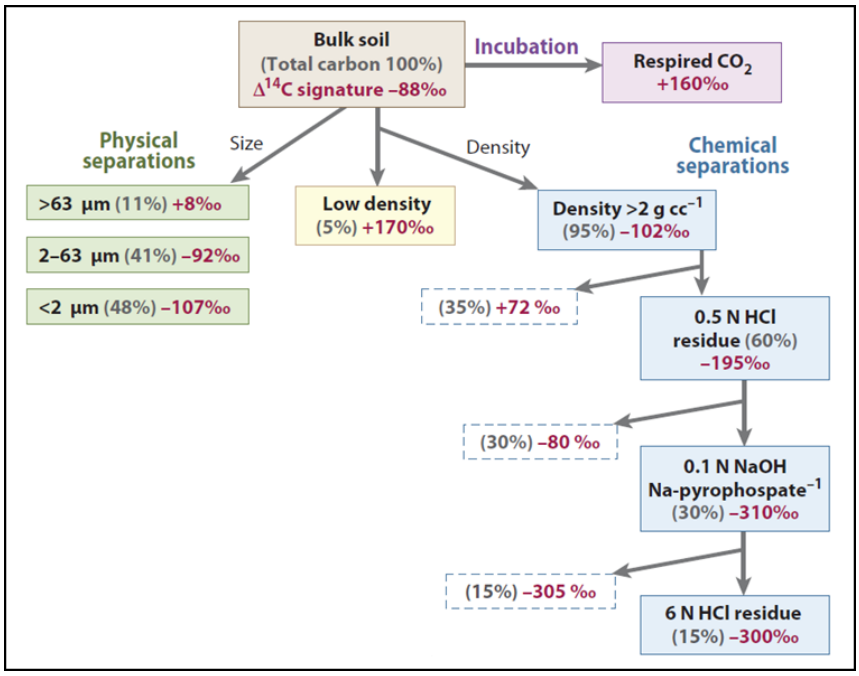


Figure 2.7. The scheme by Trumbore (2009), reports an example of changes in radiocarbon content of soil organic matter. The data showed in figure are from Trumbore & Zheng (1996) (Oxisols, 30-35 cm depth). The value in red are the signature of ^{14}C of the organic C for the different SOM fraction and for SOM during each steps of fractionation procedure, referred to size and density physical fractionation methods.

The soil organic C fraction resistant to the hydrolyses comprises 10-35% of the initial mineral associated SOC as reported by Helfrich et al. (2007) in relation to the kind of soil and/or to physical protection within microaggregates and/or strength of chemical hydrolyses applied, for example the author observed a strong correlation of C concentration in the hydrolysis-resistant fraction with initial C content. Despite it is evident that the interaction with soil minerals affects the C stabilization in soil and consequently its mean residence time and age, sill it is unclear how this interaction occurs, in term of chemistry and structure of C compounds involved, strength of their interaction with soil surfaces (Mikutta et al., 2006; Sollins et al., 2006). For example Torn et al. (1997) observed a very long (>50000 years) MRT for the large amounts of C associated with allophane that represent in soils developed from basalt material the initial weathering products; while in soils dominated by secondary weathering products such as kaolinite and iron and aluminum oxides contained less and

significantly younger C (thousand to tens of thousands of years). Similarly Masiello et al. (2004) measured in soils developed on sedimentary parent material a MRT longer (thousands to tens thousands of years) for C associated with poorly crystalline or nanocrystalline primary weathering products (ferrihydrite, allophane, imogolite) than the C associated with secondary weathering products such as crystalline clay (thousands of years).

2.5. Focusing on: *what determines the SOM recalcitrance?*

About the concept and term "recalcitrance" still controversial opinions existent. Compounds that are classified as recalcitrant or 'stable' are assumed to comprise a much greater proportion of the total soil carbon pool than those that are classified as labile (Davidson & Janssens, 2006). Historically terms like molecular recalcitrance, intrinsic-chemical recalcitrance, or resistant carbon, were employed to define the capacity of some organic compounds in biosphere to resist to microbial attack more than other ones (Himmel & Picataggio, 2009), and for example "recalcitrant-organic matter" and "refractory-organic matter" were usually utilized as synonymous. Now it is possible to affirm that, the term refractory denominate an acquired property of a material, such as the thermally altered forms of organic matter like charcoal, while recalcitrance is more appropriate to underline the behavior of a compound like the resistance as an active opposition (Kleber, 2010). Mayer (2004) proposed that the recalcitrance mainly accounts for protection on shorter time scales, and could be distinguished in primary recalcitrance (referred to plant litter and their molecular characteristics) and secondary recalcitrance (referred to microbial products, molecular aggregates and charred materials) (Lützow et al., 2006). Secondary recalcitrance become an important, sometimes the major, mechanism involved in the persistence of organic matter in soil next to interactions, accessibility, climatic-stabilization and facultative non-utilization by the decomposer community (Ekschmitt et al., 2008; Sollins et al., 1996; Trumbore, 2009). Instead primary

recalcitrance is often identifiable with chemical recalcitrance of plant litter materials, that affects their decomposition. The chemical compounds of plant residues are characterized by a large variety of organic components: polysaccharides, lignin, proteins, polyphenols, lipids, waxes, cutin and suberin. Their molecular properties, such as size, polarity, ether-bridges, quaternary C-atoms, (Ottow, 1997), influenced the decomposition rates. The polymers most resistant to degradation contain aromatic rings like lignin and can accumulate during the initial phases of decomposition. The decomposition of this structure requires strong oxidation agent and only some soil microorganism are able to mineralize them, for example in the case of lignin microorganisms involved in its decomposition are white-rot fungi (Hammel, 1997). Moreover the lignin to N ratio is also utilized as indicator of chemical recalcitrance distinguishing plant residues whit high lignin/N ratio as difficult to degrade from those more easily degraded with a lower lignin/N ratio (Moore et al., 1999; Tietema & Wessel, 1992). However, some literature findings report that lignin is altered relatively quickly and does not appear to be stabilized in the long term in any soil fraction, for example it was observed an increase in $\delta^{13}C$ value with soil depth compared with litter in contrast with the idea of a selective preservation of lignin because it is depleted in ^{13}C compared to plant tissues (Melillo et al., 1989; Nadelhoffer & Fry, 1994), it was found a lignin concentration in agricultural topsoil very small (Kögel-Knabner, 2000), moreover ^{13}C-NMR-analyses showed that the macromolecular compounds decreased with soil depth accompanied also by a decrease in OM aromacity (Rumpel et al., 2002). Therefore this empirical observations allow to affirm that primary recalcitrance is important in the first stage of litter decomposition and that the persistence of unaltered plant components is not due only to structural characteristics but also to other stabilization mechanisms, concluding that chemical structure alone is not sufficient to account for the extreme variation in age and turnover time of OM.

On the contrary, secondary recalcitrance, related to physical-chemical protection through the interaction with mineral and physical protection from microbial decomposers through aggregation, play a very important role in SOM stabilization. In particular the inaccessibility to decomposer and organo-mineral interaction are the dominant mechanisms of stabilization that determine not only the long turnover times in fraction during later decomposition phase, but also the stabilization of soluble water exudates. In fact plant materials deposited by roots in soil are classified or as soluble water exudates (sugar, amino acids, organic acids) or water insoluble materials (cell walls, sloughed-off materials, root debris). The first ones are generally considered labile compounds that are mineralized within a few hours after release (Chabbi et al., 2001), even if their negative charge makes these molecules rapidly sorbable by mineral phase by cation bonding (Jones, 1998). As concern the physical protection of OM in soil aggregates (macro and microaggregates), it is determined by the inaccessibility of fine pores to microbial decomposers and their anoxic condition (Six et al., 2002). In fact microaggregates are smaller than 250 μm and the more stable are comprised between 2-20 μm (Krull et al., 2003), moreover the microbes are unable to penetrate the pore smaller than 3 μm (Kilbertus, 1980) and 10 μm of pore size determines the boundary between free drainage water and capillary water (Kirkham & Powers, 1972), so in pore with a diameter smaller than 10 μm anoxic condition could be recorded, hindering the microbial decomposition and favouring the OM accumulation. This brief and simplified description of the mechanisms involved in primary and secondary recalcitrance allows to summarize this remarks: the decrease in the rate of decomposition over time is determined by the combination and interaction of different mechanisms of SOM stabilization, some of them are independent of molecular properties of organic matter such as the incorporation of C in the protective structure of soil, and some other are based on the chemical structure, such as the production of more recalcitrant organic material derived from the decomposition processes of selected compounds (Six et al., 2002; Sollins et al.,

1996), however due to the complexity of the soil mechanisms involved in C dynamic, a clear and net separation of each kind of mechanism is difficult or impossible.

References

Bahri, H., Rasse, D.P., Rumpel, C., Dignac, M.F., Bardoux, G., Mariotti, A. 2008. Lignin degradation during a laboratory incubation followed by ^{13}C isotope analysis. *Soil Biology and Biochemistry*, **40**(7), 1916-1922.

Besnard, E., Chenu, C., Balesdent, J., Puget, P., Arrouays, D. 1996. Fate of particulate organic matter in soil aggregates during cultivation. *European Journal of Soil Science*, **47**(4), 495-503.

Buurman, P., Peterse, F., Almendros Martin, G. 2007. Soil organic matter chemistry in allophanic soils: a pyrolysis-GC/MS study of a Costa Rican Andosol catena. *European Journal of Soil Science*, **58**(6), 1330-1347.

Castanha, C., Trumbore, S., Amundson, R. 2008. Methods of separating soil carbon pools affect the chemistry and turnover time of isolated fractions. *Radiocarbon*, **54(1):** 83-97.

Chabbi, A., Hines, M.E., Rumpel, C. 2001. The role of organic carbon excretion by bulbous rush roots and its turnover and utilization by bacteria under iron plaques in extremely acid sediments. *Environmental and Experimental Botany*, **46**(3), 237-245.

Christensen, B.T. 1992. Physical fractionation of soil and organic matter in primary particle size and density separates. In: *Advances in soil science*, Springer, pp. 1-90.

Christensen, B.T. 2001. Physical fractionation of soil and structural and functional complexity in organic matter turnover. *European Journal of Soil Science*, **52**(3), 345-353.

Cotrufo, M.F., Wallenstein, M.D., Boot, C.M., Denef, K., Paul, E. 2013. The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Global Change Biology*, **19**(4), 988-995.

Davidson, E.A., Janssens, I.A. 2006. Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature*, **440**(7081), 165-173.

Dijkstra, P., Thomas, S.C., Heinrich, P.L., Koch, G.W., Schwartz, E., Hungate, B.A. 2011. Effect of temperature on metabolic activity of intact microbial communities: Evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. *Soil Biology and Biochemistry*, **43**(10), 2023-2031.

Dümig, A., Häusler, W., Steffens, M., Kögel-Knabner, I. 2012. Clay fractions from a soil chronosequence after glacier retreat reveal the initial evolution of organo–mineral associations. *Geochimica et Cosmochimica Acta*, **85**, 1-18.

Ekschmitt, K., Kandeler, E., Poll, C., Brune, A., Buscot, F., Friedrich, M., Gleixner, G., Hartmann, A., Kästner, M., Marhan, S. 2008. Soil-carbon preservation through habitat constraints and biological limitations on decomposer activity. *Journal of Plant Nutrition and Soil Science*, **171**(1), 27-35.

Falloon, P., Smith, P. 2000. Modelling refractory soil organic matter. *Biology and fertility of soils*, **30**(5-6), 388-398.

Hammel, K. 1997. Fungal degradation of lignin. *Driven by nature: plant litter quality and decomposition*, 33-45.

Helfrich, M., Flessa, H., Mikutta, R., Dreves, A., Ludwig, B. 2007. Comparison of chemical fractionation methods for isolating stable soil organic carbon pools. *European Journal of Soil Science*, **58**(6), 1316-1329.

Himmel, M.E., Picataggio, S.K. 2009. Our challenge is to acquire deeper understanding of biomass recalcitrance and conversion. *Biomass recalcitrance: Deconstructing the plant cell wall for bioenergy*, 1-6.

Jenny, H. 1980. The soil resource: origin and behavior. *Springer Science & Business Media.*

Jones, D., Edwards, A. 1998. Influence of sorption on the biological utilization of two simple carbon substrates. *Soil Biology and Biochemistry*, **30**(14), 1895-1902.

Jones, D.L. 1998. Organic acids in the rhizosphere–a critical review. *Plant and soil*, **205**(1), 25-44.

Kaiser, K., Zech, W. 1998. Soil dissolved organic matter sorption as influenced by organic and sesquioxide coatings and sorbed sulfate. *Soil Science Society of America Journal*, **62**(1), 129-136.

Kilbertus, G. 1980. Study of the Microhabitat Contents in the Soil Aggregates; Their Relation with the Bacterial Biomass and the Total of Procaryotes Present. *Rev. Ecol. Biol. Sol.*, **17**(4), 543-557.

Kirkham, D., Powers, W.L. 1972. *Advanced soil physics*. Wiley.

Kleber, M. 2010. What is recalcitrant soil organic matter? *Environmental Chemistry*, **7**(4), 320-332.

Kleber, M., Johnson, M.G. 2010. Chapter 3 - Advances in Understanding the Molecular Structure of Soil Organic Matter: Implications for Interactions in the Environment. in: *Advances in Agronomy*, (Ed.) L.S. Donald, Vol. Volume 106, Academic Press, pp. 77-142.

Knicker, H. 2011. Soil organic N - An under-rated player for C sequestration in soils? *Soil Biology and Biochemistry*, **43**(6), 1118-1129.

Kögel-Knabner, I. 2000. Analytical approaches for characterizing soil organic matter. *Organic Geochemistry*, **31**(7), 609-625.

Kögel-Knabner, I., Guggenberger, G., Kleber, M., Kandeler, E., Kalbitz, K., Scheu, S., Eusterhues, K., Leinweber, P. 2008. Organo-mineral associations in temperate soils: Integrating biology, mineralogy, and organic matter chemistry. *Journal of Plant Nutrition and Soil Science*, **171**(1), 61-82.

Krull, E.S., Baldock, J.A., Skjemstad, J.O. 2003. Importance of mechanisms and processes of the stabilisation of soil organic matter for modelling carbon turnover. *Functional Plant Biology*, **30**(2), 207-222.

Lehmann, J., Solomon, D., Kinyangi, J., Dathe, L., Wirick, S., Jacobsen, C. 2008. Spatial complexity of soil organic matter forms at nanometre scales. *Nature Geosci*, **1**(4), 238-242.

Levin, I., Bösinger, R., Bonani, G., Francey, R.J., Kromer, B., Münnich, K., Suter, M., Trivett, N.B., Wölfli, W. 1992. Radiocarbon in atmospheric carbon dioxide and methane: global distribution and trends. In: *Radiocarbon after four decades*, Springer, pp. 503-518.

Levin, I., Hesshaimer, V. 2000. Radiocarbon- a unique tracer of global carbon cycle dynamics.

Lützow, M.v., Kögel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B., Flessa, H. 2006. Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions – a review. *European Journal of Soil Science*, **57**(4), 426-445.

Marzaioli, F., 2011. Assessing soil organic matter dynamics by Means of ^{14}C measurements. In: Advances in Environmental Research. Volume 17 Editor: Justin A. Daniels, pp.1-32.

Masiello, C., Chadwick, O., Southon, J., Torn, M., Harden, J. 2004. Weathering controls on mechanisms of carbon storage in grassland soils. *Global Biogeochemical Cycles*, **18**(4).

Melillo, J.M., Aber, J.D., Linkins, A.E., Ricca, A., Fry, B., Nadelhoffer, K.J. 1989. Carbon and nitrogen dynamics along the decay continuum: plant litter to soil organic matter. in: *Ecology of Arable Land—Perspectives and Challenges*, Springer, pp. 53-62.

Mayer, L.M. 2004. The inertness of being organic. *Marine Chemistry*, *92*-135

Mikutta, R., Kaiser, K. 2011. Organic matter bound to mineral surfaces: Resistance to chemical and biological oxidation. *Soil Biology and Biochemistry*, **43**(8), 1738-1741.

Mikutta, R., Kleber, M., Torn, M.S., Jahn, R. 2006. Stabilization of soil organic matter: association with minerals or chemical recalcitrance? *Biogeochemistry*, **77**(1), 25-56.

Moni, C., Derrien, D., Hatton, P.-J., Zeller, B., Kleber, M. 2012. Density fractions versus size separates: does physical fractionation isolate functional soil compartments?

Moore, T., Trofymow, J., Taylor, B., Prescott, C., Camire, C., Duschene, L., Fyles, J., Kozak, L., Kranabetter, M., Morrison, I. 1999. Litter decomposition rates in Canadian forests. *Global Change Biology*, **5**(1), 75-82.

Nadelhoffer, K., Fry, B. 1994. Nitrogen isotope studies in forest ecosystems. *Stable isotopes in ecology and environmental science. Blackwell, Oxford*, **316**.

Naegler, T., Levin, I. 2006. Closing the global radiocarbon budget 1945–2005. *Journal of Geophysical Research: Atmospheres*, **111**(D12).

Oades, J. 1988. The retention of organic matter in soils. *Biogeochemistry*, **5**(1), 35-70.

Oades, J., Vassallo, A., Waters, A., Wilson, M. 1987. Characterization of organic matter in particle size and density fractions from a red-brown earth by solid state ^{13}C NMR. *Soil Research*, **25**(1), 71-82.

Olk, D.C., Gregorich, E.G. 2006. Overview of the symposium proceedings,"Meaningful pools in determining soil carbon and nitrogen dynamics". *Soil Science Society of America Journal*, **70**(3), 967-974.

Ottow, J. 1997. Abbaukinetik und Persistenz von Fremdstoffen in Böden, Fischer, Stuttgart, pp. 97-138.

Paul, E.A. 2014. Soil microbiology, ecology and biochemistry. *Academic press.*

Paul, E.A., Morris, S.J., Conant, R.T., Plante, A.F. 2006. Does the acid hydrolysis–incubation method measure meaningful soil organic carbon pools? *Soil Science Society of America Journal*, **70**(3), 1023-1035.

Piccolo, A. 2002. The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. *Advances in Agronomy*, **75**, 57-134.

Randerson, J., Enting, I., Schuur, E., Caldeira, K., Fung, I. 2002. Seasonal and latitudinal variability of troposphere $\Delta^{14}CO_2$: Post bomb contributions from fossil fuels, oceans, the stratosphere, and the terrestrial biosphere. *Global Biogeochemical Cycles*, **16**(4).

Rasse, D.P., Rumpel, C., Dignac, M.-F. 2005. Is soil carbon mostly root carbon? Mechanisms for a specific stabilisation. *Plant and soil*, **269**(1-2), 341-356.

Reimer, P.J., Baillie, M.G., Bard, E., Bayliss, A., Beck, J.W., Bertrand, C.J., Blackwell, P.G., Buck, C.E., Burr, G.S., Cutler, K.B. 2004. IntCal04 terrestrial radiocarbon age calibration, 0-26 cal kyr BP.

Rumpel, C., Kögel-Knabner, I., Bruhn, F. 2002. Vertical distribution, age, and chemical composition of organic carbon in two forest soils of different pedogenesis. *Organic Geochemistry*, **33**(10), 1131-1142.

Saggar, S., Parshotam, A., Sparling, G., Feltham, C., Hart, P. 1996. ^{14}C-labelled ryegrass turnover and residence times in soils varying in clay content and mineralogy. *Soil Biology and Biochemistry*, **28**(12), 1677-1686.

Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., Kleber, M., Kogel-Knabner, I., Lehmann, J., Manning, D.A.C., Nannipieri, P., Rasse, D.P., Weiner, S., Trumbore, S.E. 2011. Persistence of soil organic matter as an ecosystem property. *Nature*, **478**(7367), 49-56.

Schulze, K., Borken, W., Muhr, J., Matzner, E. 2009. Stock, turnover time and accumulation of organic matter in bulk and density fractions of a Podzol soil. *European Journal of Soil Science*, **60**(4), 567-577.

Six, J., Conant, R., Paul, E.A., Paustian, K. 2002. Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and soil*, **241**(2), 155-176.

Six, J., Elliott, E., Paustian, K. 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. *Soil Biology and Biochemistry*, **32**(14), 2099-2103.

Six, J., Frey, S.D., Thiet, R.K., Batten, K.M. 2006. Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems. *Soil Science Society of America Journal*, **70**(2).

Six, J., Jastrow, J.D. 2002. Organic matter turnover. *Encyclopedia of soil science. Marcel Dekker, New York*, 936-942.

Six, J., Schultz, P., Jastrow, J., Merckx, R. 1999. Recycling of sodium polytungstate used in soil organic matter studies. *Soil Biology and Biochemistry*, **31**(8), 1193-1196.

Sollins, P., Homann, P., Caldwell, B.A. 1996. Stabilization and destabilization of soil organic matter: mechanisms and controls. *Geoderma*, **74**(1–2), 65-105.

Sollins, P., Swanston, C., Kleber, M., Filley, T., Kramer, M., Crow, S., Caldwell, B.A., Lajtha, K., Bowden, R. 2006. Organic C and N stabilization in a forest soil: evidence from sequential density fractionation. *Soil Biology and Biochemistry*, **38**(11), 3313-3324.

Sposito, G., Skipper, N.T., Sutton, R., Park, S.-h., Soper, A.K., Greathouse, J.A. 1999. Surface geochemistry of the clay minerals. *Proceedings of the National Academy of Sciences*, **96**(7), 3358-3364.

Strosser, E. 2010. Methods for determination of labile soil organic matter: an overview. *Journal of Agrobiology*, **27**(2), 49-60.

Stuiver, M., Polach, H.A. 1977. Discussion; reporting of C-14 data. *Radiocarbon*, **19**(3), 355-363.

Sutton, R., Sposito, G. 2005. Molecular structure in soil humic substances: the new view. *Environmental Science Technology*, **39**(23), 9009-15.

Tietema, A., Wessel, W.W. 1992. Gross nitrogen transformations in the organic layer of acid forest ecosystems subjected to increased atmospheric nitrogen input. *Soil Biology and Biochemistry*, **24**(10), 943-950.

Torn, M., Swanston, C., Castanha, C., Trumbore, S. 2009. Storage and turnover of organic matter in soil. *Biophysico-chemical processes involving natural nonliving organic matter in environmental systems. Wiley, Hoboken*, 219-272.

Torn, M.S., Trumbore, S.E., Chadwick, O.A., Vitousek, P.M., Hendricks, D.M. 1997. Mineral control of soil organic carbon storage and turnover. *Nature*, **389**(6647), 170-173.

Trumbore, S. 2000. Age of soil organic matter and soil respiration: radiocarbon constraints on belowground C dynamics. *Ecological Applications*, **10**(2), 399-411.

Trumbore, S. 2009. Radiocarbon and soil carbon dynamics. *Annual Review of Earth and Planetary Sciences*, **37**, 47-66.

Trumbore, S.E. 1993. Comparison of carbon dynamics in tropical and temperate soils using radiocarbon measurements. *Global Biogeochemical Cycles*, **7**(2).

Trumbore, S.E. 1997. Potential responses of soil organic carbon to global environmental change. *Proceedings of the National Academy of Sciences*, **94**(16), 8284-8291.

Trumbore, S.E., Zheng, S. 1996. Comparison of fractionation methods for soil organic matter 14C analysis. *Radiocarbon*, **38**(2), 219-229.

von Lützow, M., Kögel-Knabner, I., Ekschmitt, K., Flessa, H., Guggenberger, G., Matzner, E., Marschner, B. 2007. SOM fractionation methods: relevance to functional pools and to stabilization mechanisms. *Soil Biology and Biochemistry*, **39**(9), 2183-2207.

Zhang, D., Hui, D., Luo, Y., Zhou, G. 2008. Rates of litter decomposition in terrestrial ecosystems: global patterns and controlling factors. *Journal of Plant Ecology*, **1**(2), 85-93.

Chapter 3- Estimate of C sequestration in Italian olive groves under different managements

3.1. Introduction

In recent years, it has been greatly increased the awareness that a correct management of the agro-ecosystems plays a key role for a global policy on sustainable development and climate protection. Cropland soils are estimated to be the largest biospheric source of C in Europe each year (Janssens et al., 2003), because globally the cultivation and soil disturbance are generally considered responsible for a C loss from the soils included between 40 and 90 PgC (Lal, 1999). Only in 1990s it has been estimated that land use change emitted 1.6± 0.8 Pg C y^{-1} to the atmosphere (IPCC 2001). In Italian croplands the loss of C has been calculated to be about 0.2-0.5 MgC $ha^{-1}y^{-1}$ (Gardi & Sconosciuto, 2007; Morari et al., 2006).

In general, soil organic carbon (SOC) tends to be lost when natural ecosystem, like grasslands or forests, are converted to croplands or if are subjected to other kinds of management; on the contrary, the SOC tends to increase when the native vegetation or natural conditions are restored (Smith, 2007). However, management practices that increase C inputs, or reduce C losses, are helpful to maintain the SOC levels in managed lands, preventing its depletion. Lal (2004; 2011) estimated the global C sequestration potential for cropland soils around 0.9 ± 0.3 PgC y^{-1}. The soil organic C stocks in Italian cropland, under different land use, has been recently estimated by Chiti et al. (2012), reporting the following mean values for the subcategories analysed: 63.3 MgC ha^{-1} in rice fields, 53.1 MgC ha^{-1} in arable lands, and, for tree crop systems, 51.5 MgC ha^{-1} in olive groves, 48.9 MgC ha^{-1} in agroforestry systems, 44.1 MgC ha^{-1} in orchards, and 41.9 MgC ha^{-1} in vineyards; with a total C storage in the upper 30 cm of the whole cropland categories amounting to ~ 490 TgC, that are about 17% of the total SOC in the upper 50 cm of Italian soils (2900 TgC, Fantappiè et al., 2010). As reviewed in literature (Freibauer et al., 2004; Lal, 2011; Smith et al.,

2007), the C sequestration potential due to the adoption of specific management practices in the agricultural activity (i.e. rotation, deintensification, reduced tillage, set aside in cropland or fire management, introduction of leguminous in grazing land management) range from about 0.3 to 0.8 tC $ha^{-1}y^{-1}$, underlining the importance to adopt agricultural practices with low-C-impact. In the Kyoto Protocol land use and land management changes are included in the practices able to reduce the atmospheric CO_2, in particular in Article 3.3 and 3.4 afforestation, reforestation, re-vegetation, and forest, cropland and grazing management are mentioned as activities that signatory countries of the Protocol may elect for meeting its C emission reduction target. During the 17^{th} Conference of Parties, in Durban 2011, the forest management becomes mandatory, rather than voluntary, for all industrialized countries, defining a turning point in future of agriculture and forestry as a mitigation tool in fighting climate change. Also tree crops are contemplated and might represent, in theory, a relevant management to improve C sequestration in soils, but in this contest they are still under investigated (Carlisle et al., 2006; IPCC, 2007; Agnelli et al., 2014). One of the most important tree crop in Europe and in the whole Mediterranean area is olive grove.In Mediterranean area olive groves cover about 7 Mha, of which 4,1 Mha are included in European country (Eurostat, 2008) and 1,1 Mha in Italy only, that are principally distributed in areas with the most typically Mediterranean climate. Chiti et al. (2012) estimated that soil C stock in Italian olive groves is strongly affect by local climate conditions (about 42.1 MgC ha^{-1} in Mediterranean sub-continental to continental climate type, and 56.0 MgC ha^{-1} in Mediterranean sub-oceanic to oceanic climate type), and this study highlighted that with arable land, olive groves contained a significantly higher mean C stock than other agricultural categories. Therefore olive groves could be for Italy the most promising sectors for national policies of climate change mitigation. Whilst estimates of soil C stock in European olive groves or more in general in tree crops system are available (Chiti et al., 2012; Martin et al., 2011), little is known about the organic C

stability and its permanence in these agroecosystems. Being these very important aspects in order to define if really that environments are able to improve C sequestration in soils (compared to other management strategies), the adoption of an appropriate study approach is necessary to make a right evaluations. A valid method is to evaluate the OC dynamics in different organic matter pools, separated for example by a SOM fractionation that allows separation in more homogeneous pools (in term of chemical and physical properties) than bulk soil, followed by the determination of the OC distribution among the fractions and by a radiocarbon analyses (^{14}C). The result is the definition of the organic C distribution among the SOM fractions with different stability and of C permanence time in each pool. This approach, often applied in natural ecosystems, could be also adapted to managed ones in order to evaluate the impacts of land use change or land management on soil C stock or on soil potential in C sequestration.

In this contest, the aim of this study was to evaluate the variations in organic C stock due to different cropping systems.

As crop systems, the tree crop of olive grove was selected, and compared to arable land in order to evaluate how the tree cropping system can contribute to the changes in soil C stock in bulk soil and in SOM fractions with different stability, both in top layer and along soil profile. The SOM wasseparated by physical-chemical fractionation procedure, and the measure of ^{14}C signature in more stable fractions was evaluated as support in the definition of C stock derived from land cover change, its stability and permanency in soil.

3.2. Materials and methods

3.2.1. Study area and soil sampling

The two study areas were both located in Deruta municipality, near Assisi town (Central Italy, Umbria region). The first one (in Monticchio hamlet) included two olive groves intensively managed with different age (7 and 30 years old, I7 and I30-

B, respectively), an olive grove super-intensively managed (7 years old, SI7), and an arable land (control). The other one (in Madonna de' Bagni hamlet) included a 30 years old intensively managed olive grove (I30-A).

The soils, constituting the studied four experimental conditions, were classified as follow (Soil Survey Staff, 2012):

- Control= Vertic Ustorthent, fine, mixed, active, calcareous, mesic;
- SI7 and I7= Vertic Haplustept, fine, mixed, active, calcareous, mesic;
- I30-A= Typic Haplustept, fine silty, mixed, calcareous,mesic
- I30-B= Typic Haplustept, sandy, mixed, calcareous, mesic;

The previous land use was characterized by crop land (cereal crop) for all the olive groves in Monticchio, while the olive grove in Madonna de'Bagni replaced a previous olive grove, with an effectively land use as tree crop longer that the actual age of the plantation. All the olive groves were under conventional management, consisting in a rotary tillage (about 20 cm deep) applied 3 or 4 times a year, to control weeds, no use of herbicides, application of mineral fertilizers, no irrigation. Moreover, after the harvest period, olive pruning residues were cut in little pieces (about 5 or 7 cm) and left on the soil surface. The differences between intensive and super-intensive management principally consisted in a different planting pattern and mineral fertilization doses. The intensively managed olive groves (I7, I30-A, I30-B) had a plant density of about 277 trees ha^{-1} (6m x 6m planting pattern), while the super-intensively managed olive groves (SI7) had about 2222 trees ha^{-1} (3m x 1.5m planting pattern). Moreover, the intensively managed olive groves only received 3,5 t ha^{-1} of urea in spring, while the super-intensively managed received 2 q ha^{-1} of urea, 1 q ha^{-1} of NPK fertilizer in spring and 1,5 q ha^{-1} of ammonium nitrate from June to September. Both in olive groves and in control, soil samples were collected in the top layer (0-10 cm depth) and along soil profiles. In the olive groves the sampling was performed in two particular positions within the field, in order to take into account the field variability: 1) under tree canopy and 2) between tree rows. In the top soil

layer four samplings for each field position were collected, with a total amount of 8 replicates for each field (Figure 3.1). Moreover, in each field position two soil profiles were opened, with a total of 4 profiles in each olive grove and 2 profiles in control field. However in I30-A site only two profiles were opened, one for each field position (1 under tree canopy + 1 between tree row).

All the soil samples were sieved at 2 mm mesh and dried at room temperature before the analyses.

3.2.2. Soil organic matter fractionation

Soil organic matter was fractionated by a physical-chemical fractionation method (Figure 3.1) (Castanha et al., 2008; Marzaioli et al., 2010; Trumbore & Zheng, 1996). Firstly, a density separation , by a sodium polytungstate solution (SPT, $Na_6(H_2W_{12}O_{40})$ d= 1,9 g cm^{-3}) was carried out to separate bulk soil in two fractions: light and heavy fractions. The first one, with a density lower than SPT solution, floated on the surface of the solution (LF); the other one, more dense, precipitated in the bottom of the tube (HF). Briefly, an amount of about 2 g of dry soil (bulk soil) was added in a tube with 30 ml of SPT solution, shaken by hand and centrifuged at 2500 rpm for 5 minutes. The floating LF was filtered on Teflon filter (0.45 µm pore diameter) placed in a vacuum filter apparatus connected with a pump. The operation was repeated more times until the complete LF removal from the soil sample. At the end of this step, both fractions were washed by distilled water to remove the salt residues. Finally both fraction were dried in oven at 70°C and then weighed. On HF a further chemical fractionation was performed with the purpose to hydrolyse the organic matter slight interacting with minerals (named *hydrolysable* fraction, HYD) and to collect, at the end of the reactions, the organic matter fraction with the stronger mineral interaction (named *recalcitrant* fraction, REC). The chemical fractionation was carried out on 700 mg of HF, and consists in three reactions. Firstly, each samples was added with about 50 ml of HCl 0.5 N solution (with the purpose to remove carbonates), than the samples were added with a basic solution of sodium

pyrophosphate and sodium hydroxide (0.1 NaOH plus 0.1 $Na_5P_2O_7$), and finally another acid reaction was carried out with a HCl 6 N solution. Each chemical treatment was applied overnight on the sample residues from the previous chemical treatment and at the end of each reaction and before the next step, the pH of each sample was neutralized with distilled water. At the end of the process, the REC was recovered and dried at 70°C.

3.2.3. Organic C and total N

The recovered LF, HF and REC fraction, and also the bulk soil, were analysed for organic C (OC) and, only bulk soil, for total N (TN) content. Samples of bulk soil and SOM fractions were pulverized: 1) by a mortar (SOM fractions, but LF was previously frozen in liquid N_2), or 2) by a knife mills provided with carbide blades (the bulk soil), to obtain a fine homogeneous powder required for OC and TN analysis and necessary to reduce the time required for bulk soil and HF to remove carbonate with the acid treatment (Caughey et al., 1995). Carbonates removal was performed by fumigation with HCl method as reported by Harris et al. (2001). Briefly, an amount of 30 mg of the ground sample of HF or bulk soil were weighed into silver capsules (9 x 5 mm) left open, then 50 µl of deionized water were added to capsulesthat were placed into a desiccator and exposed for 8 h to HCl vapor evolving from about 100 ml of 37% HCl. After this time, the samples were dried at 70°C in oven, then each silver capsule was placed into a bigger tin capsule and closed. Moreover, different amount of LF and REC (about 5 mg of LF and 30 mg REC) were weighted into tin caps. Finally the samples were analysed by flash combustion with a CNS Elemental Analyzer (Thermo FLASH1200). To determine the percentage of OC and the percentage of TN, a calibration was performed by different standards and in different amount for each SOM fraction: BBOT (72.53 %C, 6.51 %N), aspartic acid (36.09 %C, 10.52 %N).

The organic C content of HYD fraction was determined as mass balance between the HF and REC organic C content.

3.2.4. SOC and TN stock

The soil organic C (SOC) and total N (TN) stocks were calculated for soil top layer (0-10 cm) and deep horizons applying the equation:

$$Stock\ (\text{kg m}^{-2}) = concentration \times BD \times h$$

where the concentration is the OC or TN concentration expressed in g kg^{-1} soil d.w., BD is the soil bulk density (g cm^{-3}), h is the thickness in cm of the soil horizon. Applying the same equation also the organic C stocks in soil organic matter fractions were calculated.

3.2.5. Percent in modern carbon (pMC)

The soil organic matter fractions HF and REC and also the bulk soil were analyses for radiocarbon content as percent of modern carbon (pMC) by AMS, previous a graphite conversion of each samples (Figure 3.1). Samples of SOM fractions and bulk soil, weighed in tin caps in an amount calculated considering the organic carbon content of each ones able to ensure about 1 mg of carbon, were combusted by Elemental Analyser; the CO_2 produced was introduced in a capillary tube, that by-pass the chromatography column of EA, and by a flux of He, it is conducted in a cryogenic line, consisting in a tube frozen by liquid N_2 and by a vacuum system, the CO_2 is driven into quartz tubes. CO_2 was cryogenically transferred to a zinc reactor, that was composed by an external pyrex tube holding at its bottom a mixture of TiH_2 (7-10 mg) and Zn (35-40 mg) and a pyrex bacteriological tube filled with 2 mg of Fe powder (Marzaioli et al., 2008; Xu et al., 2007), also the zinc and titanium hydrate powder were separately pre-treated into vacuum sealed Pyrex tube at 360°C for 3 h, and after the filling of the Pyrex tube, the reactor tube was also pre-treated in muffle furnace (300°C for 1h).

The graphitization process was carried out in muffle furnace at 560°C for 8 h. Finally the graphite was pounded into 1 mm Al standard cathode and analysed at accelerator mass spectrometry (AMS) according to Terrasi et al. (2008), with processed blanks

and standards as oxalic acid. By the comparison with the nominal isotopic pMC value of the standards, it was possible to calculate the pMC of the sample. Measured radiocarbon value were expressed as pMC:

$$\mathrm{pMC} = \frac{\left(\frac{^{14}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{sample}(-25‰)}}{0.95\left(\frac{^{14}\mathrm{C}}{^{12}\mathrm{C}}\right)_{\mathrm{OX1}(-19‰)}} x100$$

3.2.6. Mean annual C sequestration rates

In order to evaluate the mean annual C sequestration in stable SOM fractions for each experimental condition, a model that simulates the organic C dynamics in soil was developed and applied. The most frequently utilized models were widely reviewed and discussed by Marzioli (2011). These models allow the calculation of MRT (or decomposition constant) of each fraction considering the amount of C stock and ^{14}C measured as input data to evaluate the annual soil C sequestration.

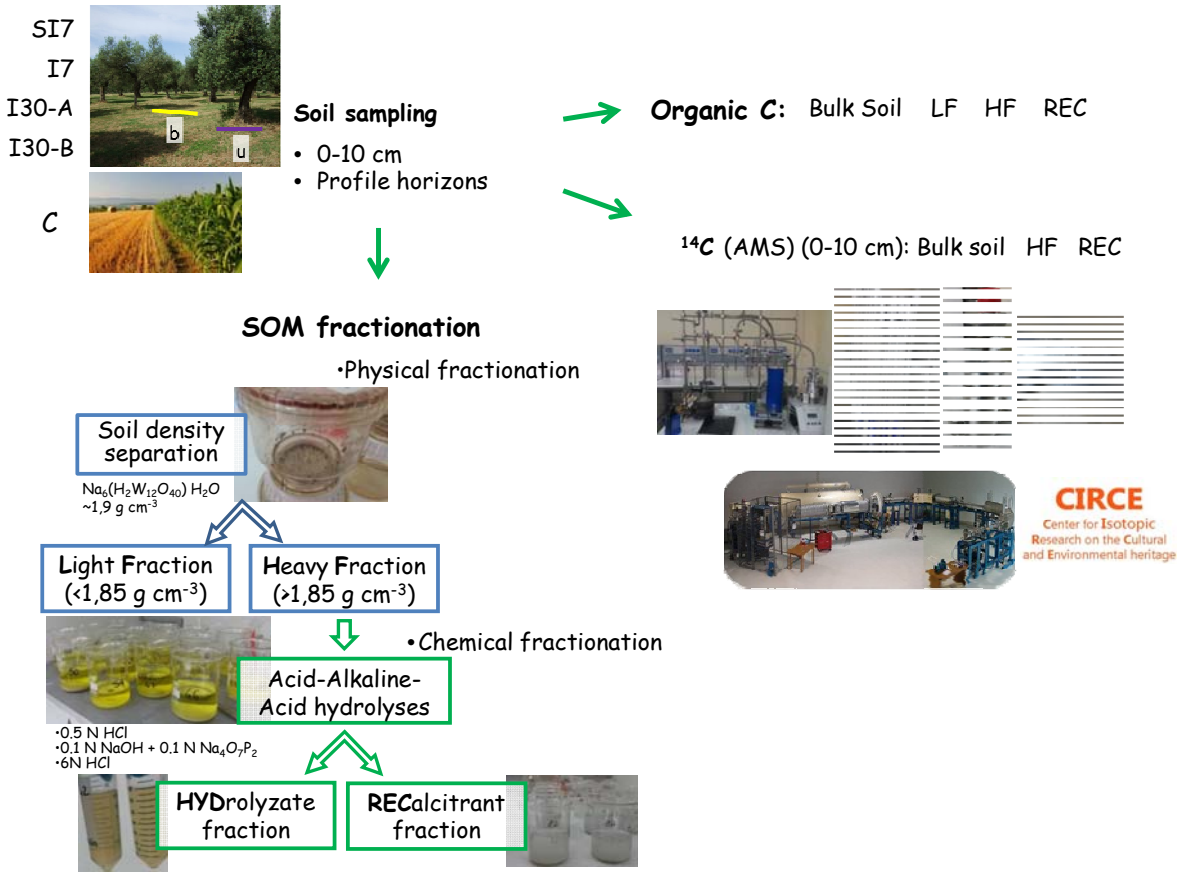


Figure 3.1 Schematic representation of soil samplings and laboratory analyses performed.
C= control; SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A in Madonna de'Bagni, -B in Monticchio).

3.2.6. Statistical analysis

The mean values and standard deviations (or standard errors) of the field replicates were calculated for each parameter analysed, such as OC and TN in bulk soil and OC in SOM fractions. For soil top layer were selected:4 replicates between tree row and 4 under three canopy (of which 2 + 2 replicates were the top soil layers of the profiles), with a total of 8 replicates for top soil layer; moreover two replicates for each horizons for profiles in control and I30-A and 4 replicates for each horizons for profiles in other olive groves were analyzed. Significant differences among experimental conditions were tested by one way ANOVA, followed by the Student-Newman-Keuls test or the Dunnet test ($P<0.05$; SigmaPlot 12.0).

3.3. Results

In Table 3.1 the results of the soil organic C (SOC) and total N (TN) concentrations (g kg^{-1}), calculated in a 10 cm depth top layer, are reported as mean values. For each olive groves (SI7, super-intensive 7 years old; I7, intensive 7 years old; I30-A, intensive 30 years old in Madonna de' Bagni; I30-B, intensive 30 years old Monticchio) the SOC and TN concentration of olive groves are reported for both field positions, between tree rows (b) and under tree canopy (u), and also as mean value in each experimental condition. Because of in both SOC and TN concentrations no significant difference was observed between the two field positions in each experimental condition, all the replicates (8 for each experimental condition) were considered together to obtain a unique mean value. The mean values of OC and TN concentrations were significantly higher in I30-A compared to control and all other olive groves (Table 3.1).

Table 3.1 Mean values (±standard deviations) of soil organic carbon (SOC) and total nitrogen (TN) concentrations measured in top layers (0-10 cm) in control and, for olive groves, in two different field positions (under tree canopy and between tree rows). Total mean values for both SOC concentration and SOC and TN stocks (Mg ha^{-1}) were shown on the right of figure. Different letters indicate statistic differences ($P<0.05$) among experimental conditions.

Top layer (0-10 cm)	**Between tree rows**		**Under tree canopy**		**Average**			
	SOC	**TN**	**SOC**	**TN**	**SOC**	**TN**	**SOC stock**	**TN stock**
	(g kg^{-1})		(g kg^{-1})		(g kg^{-1})		(Mg ha^{-1})	
Control	-	-	-	-	8.9[a] (±1.6)	1.1[a] (±0.1)	12.7[a] (±2.0)	1.6[a] (±0.1)
SI7	9.2 (±1.6)	1.2 (±0.1)	12.2 (±0.8)	1.4 (±0.0)	10.7[a] (±2.0)	1.3[a] (±0.2)	14.5[a] (±2.6)	1.9[a] (±0.2)
I7	10.8 (±1.7)	1.4 (±0.1)	11.7 (±2.0)	1.5 (±0.3)	11.3[a] (±1.8)	1.5[a] (±0.2)	15.2[a] (±3.1)	2.1[a] (±0.4)
I30-A	15.8 (±6.2)	1.7 (±0.5)	18.6 (±3.7)	2.0 (±0.3)	10.0[a] (±1.4)	1.8[a] (±0.4)	28.7[b] (±8.4)	3.1[b] (±0.7)
I30-B	8.7 (±1.0)	1.1 (±0.1)	10.9 (±0.6)	1.3 (±0.1)	17.2[b] (±5.0)	1.2[b] (±0.2)	13.3[a] (±1.0)	1.6[a] (±0.2)

SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de' Bagni; -B: in Monticchio).

The SOC and TN concentrations (g kg^{-1}) in soil top layer were converted in SOC and TN stocks by using soil bulk density data, and these stocks were calculated (for the top horizon of each profile too) considering the first 10 cm of soil (Table 3.1; Figure 3.2). SOC and TN stocks were calculated both as MgC ha^{-1} and as kg m^{-2} in order to better compare results with data available in literature. Similarly to the trend found for SOC and TN average concentrations (see Table 3.1), I30-A showed a significant higher value in both SOC and TN stocks, compared to control, the 7 years old olive groves (SI7 and I7) and the other 30 years old olive groves (I30-B). Moreover, the SOC and TN concentrations (g kg^{-1}) were measured for each horizon (with its specific thickness) of the soil profile, and the variations , are represented in Figure 3.3 a and b, respectively. The SOC concentration decreased in depth in all the four experimental conditions. The graph (Figure 3.3 a) clearly represents the significant higher concentration found in the superficial horizon (Ap) of I30-A compared to all other conditions) and shows as SOC of I30-A quickly decreased in deep, achieving at about -34 cm lower values compared to that in control, SI7 and I7 (with a significant difference found only *vs* I7). In I30-B, a quickly decrease in OC concentration was recorded yet at about 15 cm in depth, with a constant mean concentration in following below horizons. A similar behavior was also observed for total nitrogen (TN) concentrations (gN kg^{-1}) (Figure 3.3 b), however in this case, comparing all experimental conditions, no significant difference in TN concentrations was observed in Ap horizon, even if, as reported above (Table 3.1), TN concentrations calculated in the top 10 cm of soil showed that a higher TN value was measured in I30-A compared to all other experimental conditions. This apparent contrast was due to the higher number of replicates available for the top layer (0-10 cm), compared to the number of replicates for top horizons of soil profiles(i.e. 2 for control and 4 for olive groves). However, also in this case, at about 30 cm depth, significantly higher TN concentrations in control and I7 was observed compared to I30-A. A different behavior in I30-B was also observed for TN concentration, showing in deeper

horizons the lowest values compared to all other experimental conditions. Finally, no great difference among all experimental conditions was observed for C/N ratio along soil profiles (Figure 3.3 c), but only at about 30 cm in depth a significantly very low value was measured for I30-B.

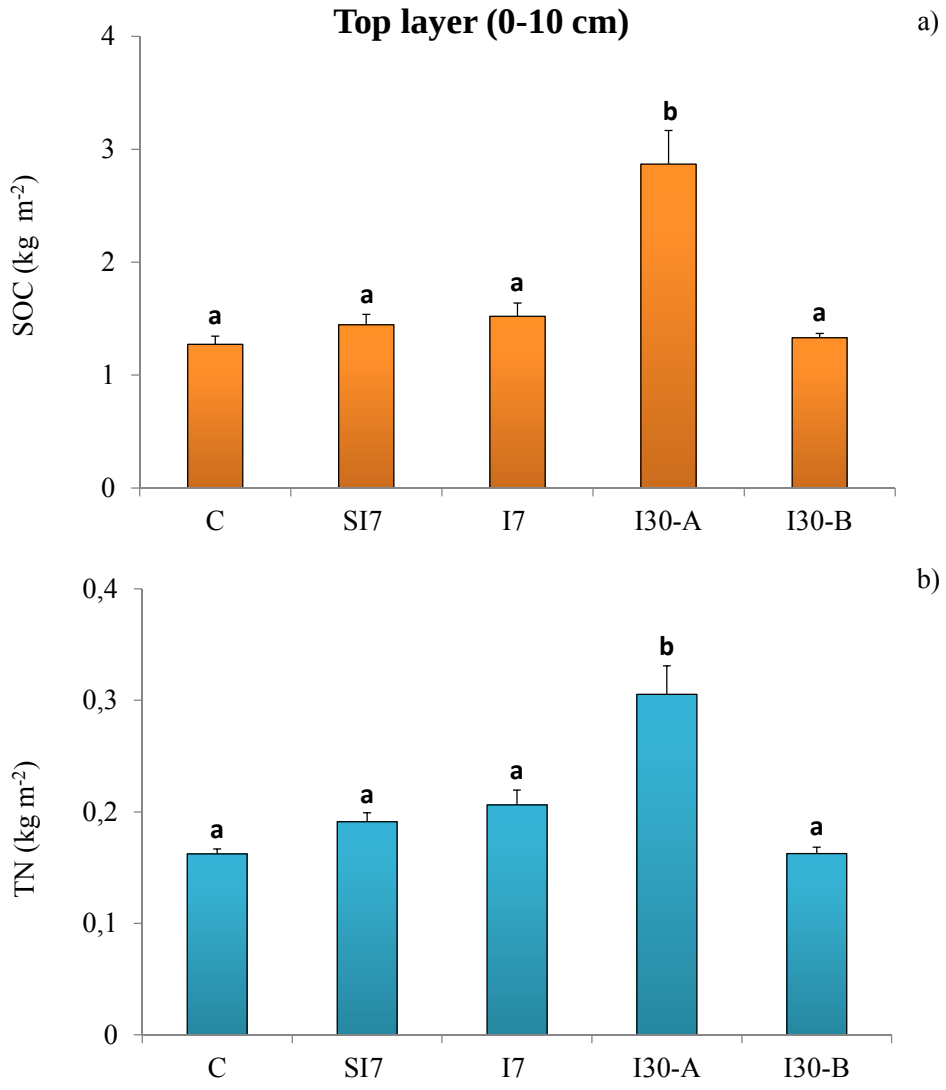


Figure 3.2 Mean values (+ standard errors) of SOC stocks (a) and TN stocks (b) (kg m^{-2}) in soil top layer (0-10 cm). Different letters on bars indicate significant differences ($P<0.05$) among experimental conditions.
C= control; SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni; -B: in Monticchio).

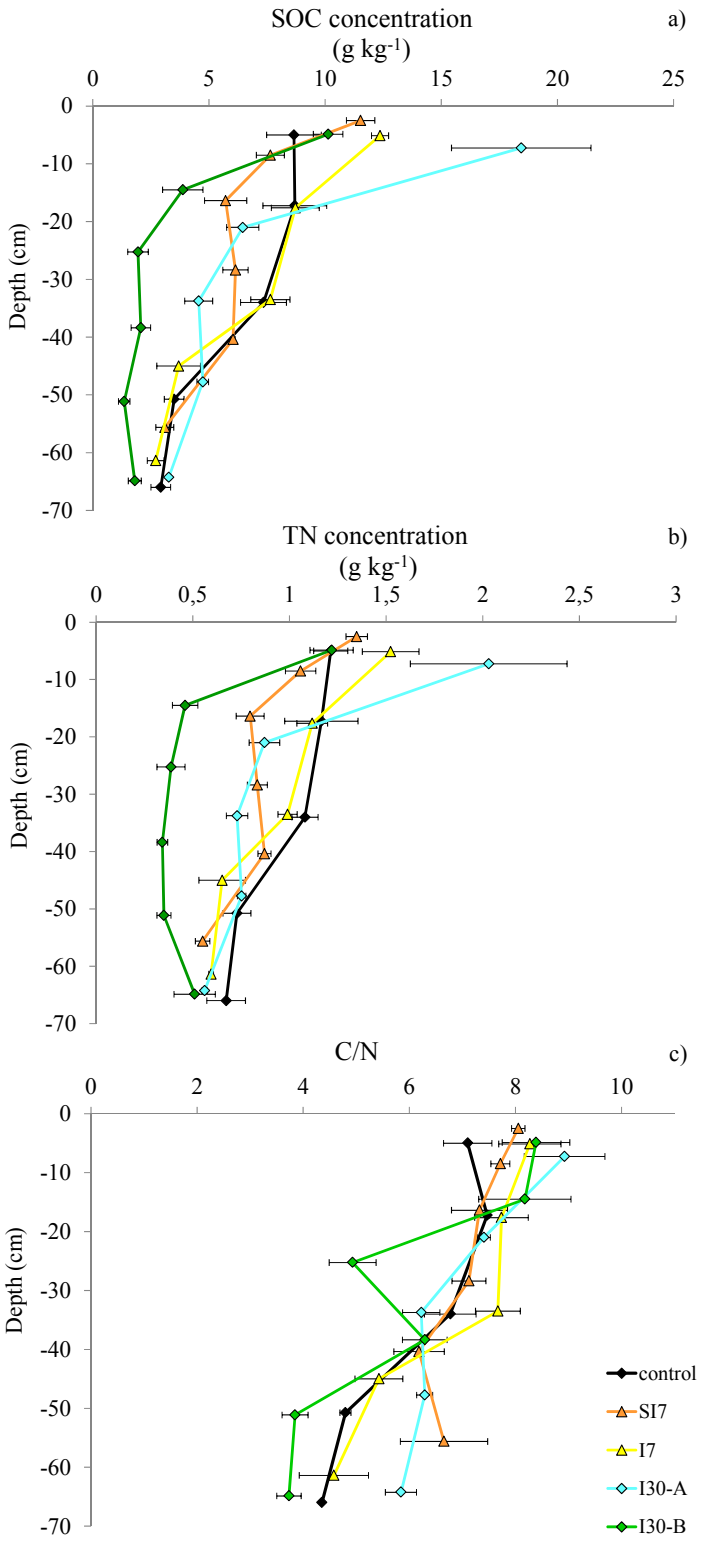


Figure 3.3 Mean values (±standard errors) of soil organic carbon (SOC) (a) and total nitrogen (TN) (b) concentrations and C/N ratio (c) at different depth along soil profile.
SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni; -B: in Monticchio).

The SOC and TN stocks (kg m^{-2}) were also calculated at different intervals of depth, respecting the horizon differentiations and their specific thickness, in order to evaluate the distribution of the stocks along soil profile (Figure 3.4 a and b). Similarly to that was reported above, the highest SOC stock in Ap horizon was measured in I30-A experimental condition, but a quick reduction in depth (already from the second horizon) was observed compared to other conditions (control, SI7, I7) that, on the contrary, showed a deep accumulation, and at about 30 cm in depth the SOC stocks in control and I7 were significantly higher compared to I30-A. Instead, at about 15 cm in depth, SI7 showed a significant lower value in SOC stock compared to control. In general, in the deeper horizons, all the olive groves followed a similar trend. The distribution in depth of the SOC stocks underlined that the OC accumulation in I30-A was strictly related to upper horizon. The two 30 years old olive groves showed for SOC stock a similar trend along soil profile, without no accumulation in deep, nevertheless the I30-A experimental condition always showed significantly higher value than I30-B. A similar results was also observed for TN stock, that appeared significantly higher in the top horizon of I30-A site compared to top horizons of all other olive grove and control sites, and that showed an increase in control and I7 sites at 30 cm in depth only whereas in SI7 site the increase was shown at about 40 cm in depth.

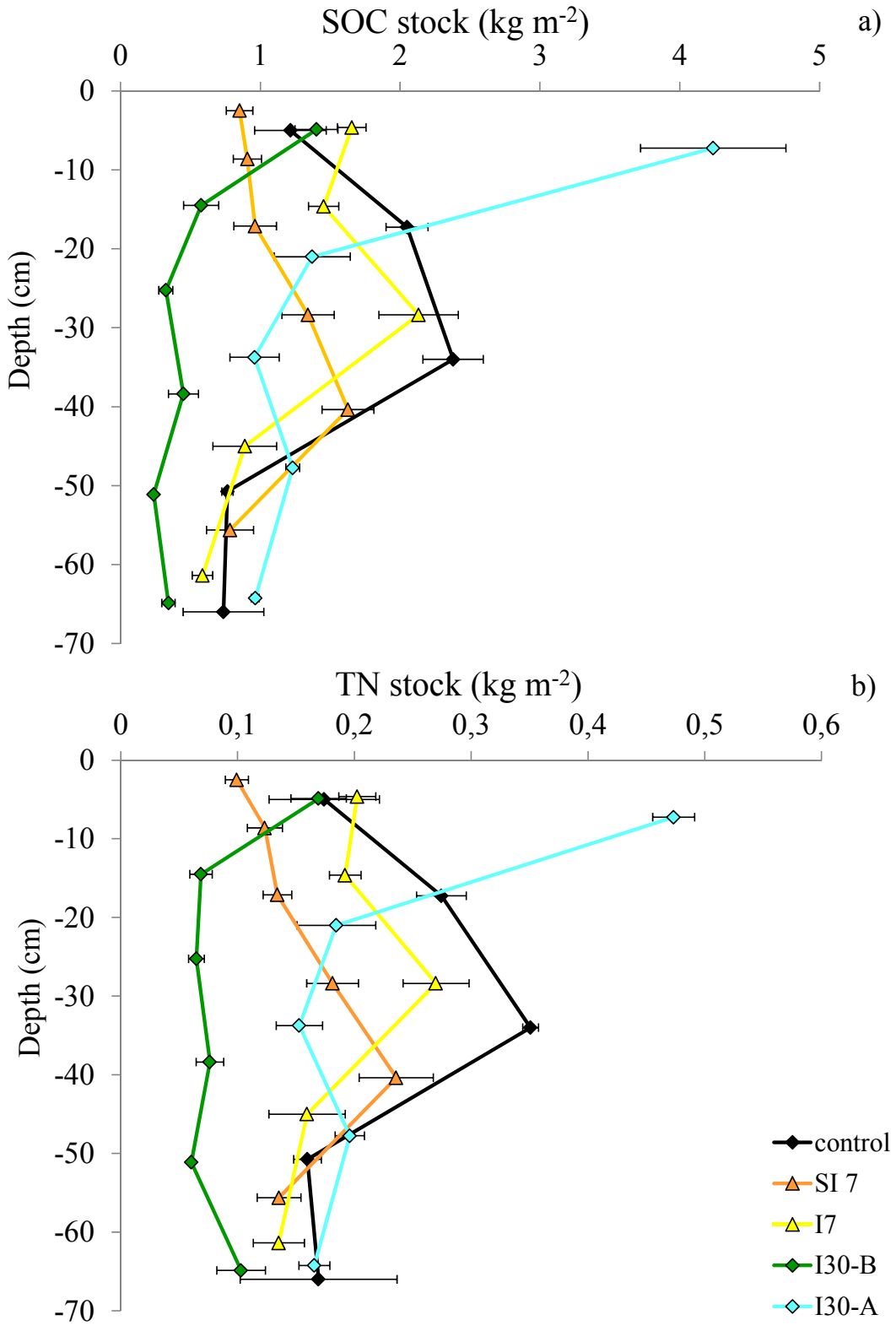


Figure 3.4 Mean values (±standard errors) of soil organic carbon stock (SOC) (a) and total nitrogen stock (TN) (b) at different depth along soil profile.
SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

The total SOC and TN stocks in bulk soil, calculated as cumulative amount of SOC and TN along the profile, are reported in Figure 3.5 a and b.

The results of the total SOC and TN stocks were converted in MgC ha^{-1} and MgN ha^{-1} and also calculated for the top 0-30 cm in order to compare our results with those reported in literature (Table 3.2).

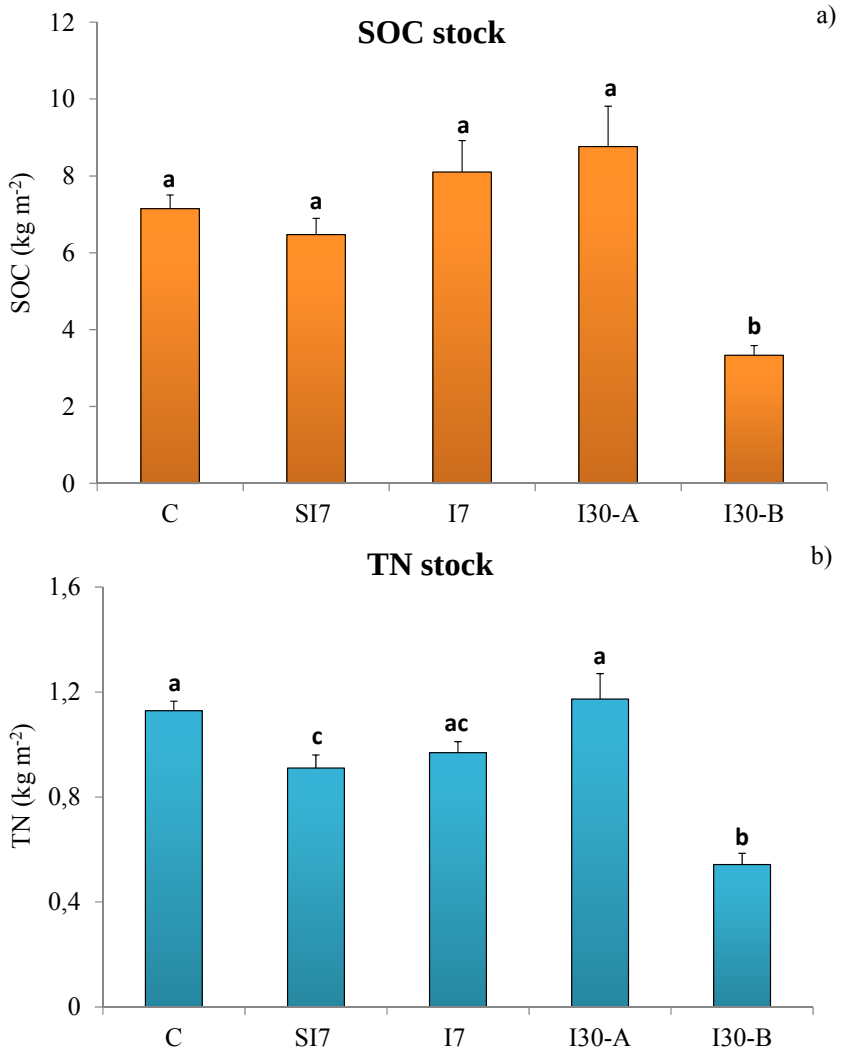


Figure 3.5 Mean values and standard errors of soil organic carbon stocks (SOC) (a) and total nitrogen stocks (TN) (b) as cumulative values of the whole profile. Different letters indicate significant differences ($P<0.05$) among experimental conditions.
C= control; SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

Table 3.2 Mean values (±standard deviations) of soil organic carbon (SOC) and total nitrogen (TN) stocks (Mg ha^{-1}) in 0-30 cm depth, total values of SOC and TN in the whole profile for each experimental condition, and mean clay content in horizons until 30 cm depth. Different letter indicate statistic differences ($P<0.05$) among experimental conditions.

		SOC stock		TN stock	
	Clay	Mg ha^{-1} (30 cm)	Mg ha^{-1} (whole profile)	Mg ha^{-1} (30 cm)	Mg ha^{-1} (whole profile)
Control	36.8%	40.0^{a} (±4.4)	71.5^{a} (±5.1)	5.5^{ac} (±0.4)	11.3^{a} (±0.5)
SI7	40.4%	35.0^{a} (±5.2)	64.7^{a} (±8.4)	4.6^{a} (±0.4)	9.1^{c} (±1.0)
I7	41.9%	43.1^{a} (±10.6)	81.0^{a} (±16.4)	5.2^{ac} (±1.1)	9.7^{ac} (±0.8)
I30-A	28.0%	53.7^{a} (±7.8)	87.7^{a} (±14.8)	6.8^{c} (±1.1)	11.7^{a} (±1.4)
I30-B	9.0%	22.1^{b} (±5.4)	33.3^{b} (±5.0)	3.0^{b} (±0.5)	5.4^{b} (±0.9)

SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A in Madonna de'Bagni, -B in Monticchio).

The difference observed above in soil top layer of I30-A (see Figure 3.2) disappeared in the total SOC stock (Figure 3.5 a), in fact, despite slightly higher values were observed in I7 and I30-A, compared to control and SI7, the differences were not significant, so SOC stocks of the whole profiles of control, SI7, I7 and I30-A experimental conditions were similar and comprised between about 7.1 and 8.0 kgC m^{-2} (control and I30-A respectively), while I30-B showed a significant lower SOC stock compared to the other experimental conditions. The SOC stocks, at 0-30 cm depth, of control, SI7, I7 and I30-A sites did not differed (Table 3.2), while I30-B showed significantly lower values than other sites. Despite I30-A was poorer in clay content, as showed by the average value reported in Table 3.2, compared to control, SI7 and I7 sites, its SOC stock was similar to other ones, while clearly appeared that the storage of C was strongly affected in I30-B by the low content in clay, showing lower values of SOM compared to the other 30 years old olive grove (I30-A). The TN stock, as cumulative value of the profile (Figure 3.4 b), in SI7 was significant

lower compared to control and I30-A, despite the type of management involving an higher application of N-fertilizers than in other sites; no differences were observed among I30-A, I7 and control , while TN stock in I30-B was significantly lower compared to other sites. The same results were yet observed at 30 cm depth (Table 3.2).

Considering the contribution of superficial SOC stock to the total amount in each experimental condition, it has to be emphasized that in I30-A and -B a very high percentage of the SOC was stored in top layer (about 33% and 40% respectively) compare to control (17.8%), SI7 (22.3%) and I7 (18.8%) sites. However, we can suppose that in 30 years old olive groves the higher contribution of the top SOC stock could be related to the specific management of this tree crop in the longer period, that promoted the superficial accumulation of SOC. On the contrary, the lower values in top soil found in control and 7 years old olive groves, highlighted a more uniform distribution of SOC with a more consisting deep accumulation, particularly in control and I7. To the different behavior probably, also contribute the past history, because of in Monticchio sites, before the olive grove plantation, the soil were cultivated as cereal crop, while in Madonna de'Bagni site the actual olive grove is a replant of a previous one, so the time of tree crop management in this latter case is higher than 30 years. Therefore, the similar superficial and more or less deep distribution of the OC (both in concentration and stock) in I7 and SI7 and control, reflect the few time of tree crop management that wasn't sufficient to produce a great variation in OC stock. However, the differences measured between the two 30 years old olive groves were certainly also due to the different percentage of clay in soils, considering that clays have a fundamental role in the physically protection of OC in soils.

The organic carbon concentration in SOM fractions (mgC g^{-1} soil d.w.) with different stability separated by fractionation method in the uppermost soil layer (0-10 cm) are reported in Table 3.3. Also in this case, no great differences in the concentration of the organic carbon were measure between the samples taken under tree canopy or in

the space between rows, and for this reason we calculated the global average. The organic C content in LF was significantly higher in I30-A compared to control and both 7 years old olive groves (I7 and SI7). The I30-A site also showed the highest OC concentration in HYD fraction compared to all other conditions, while a more homogeneous distribution of the concentration was measured in the REC fraction. However, I30-B site showed a significantly lower OC content in REC compared to I30-A and I7.

Table 3.3 Mean values (±standard deviations) of organic C (OC) in light (LF), hydrolysable (HYD) and recalcitrant (REC) fractions measured in the soil top layer (0-10 cm) of control and of olive groves for the two field positions (under tree rows and between tree canopy) and as total mean value. Different letters on apex indicate statistic differences ($P<0.05$) among experimental conditions.

Top layer (0-10 cm)	Between tree rows (mg g^{-1} d.w.)			Under tree canopy (mg g^{-1} d.w.)			Average (mg g^{-1} d.w.)		
	OC_{LF}	OC_{HYD}	OC_{REC}	OC_{LF}	OC_{HYD}	OC_{REC}	OC_{LF}	OC_{HYD}	OC_{REC}
Control	-	-	-	-	-	-	1.3^{a} (±0.7)	4.5^{a} (±0.9)	1.7^{ab} (±0.6)
SI7	1.6 (±0.6)	4.6 (±0.7)	1.5 (±0.8)	3.3 (±0.3)	5.8 (±1.3)	1.9 (±0.9)	2.5^{a} (±1.0)	5.2^{a} (±1.2)	1.7^{ab} (±0.8)
I7	2.0 (±0.7)	5.3 (±0.5)	1.9 (±0.6)	2.1 (±0.3)	6.3 (±1.0)	1.9 (±0.5)	2.1^{a} (±0.5)	5.8^{a} (±0.9)	1.9^{a} (±0.5)
I30-A	4.0 (±2.2)	9.8 (±3.1)	1.9 (±0.6)	4.3 (±2.1)	11.0 (±1.9)	2.8 (±0.4)	4.1^{b} (±2.0)	10.4^{b} (±2.5)	2.4^{a} (±0.6)
I30-B	2.8 (±0.6)	3.1 (±0.5)	1.0 (±0.2)	4.1 (±0.8)	4.8 (±1.0)	1.1 (±0.1)	3.5^{ab} (±1.0)	3.9^{a} (±1.2)	1.1^{b} (±0.2)

SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

The organic C stocks in SOM fractions of the soil top layer are reported in Figure 3.6 a. The organic C stock of I30-A appeared significantly increased in all the three SOM fractions, compared to control and other olive groves. In particular in comparison with control, in I30-A the OC in LF increased on average of 283%, in HYD fraction of 119% and in REC fraction of 60%. It was also observed a significant higher value

of OC in LF of I30-B site compared to control only, and a significant lower value of the OC in REC fraction compared to control and other olive groves. The increase in OC stock of LF (OC_{LF}) in the two older olive groves was probably due both to the major input in plant residues during the time (30 years or more) and also to the management practices adopted in all these olive groves, in which the olive pruning residues are left on field cut in little pieces. In top layer of studied sites, the HYD fraction contribute to the total SOC stock on average for 49% (control= 51%, SI7= 49.5%, I7= 53.9%, I30-A= 49.6%, I30-B= 41%) with the highest percentage in I7 site, while the REC fraction contribute on average for 16%(control= 19.4%, SI7= 19.9%, I7= 18.8%, I30-A= 13.8%, I30-B=11.9%), with highest values in control,SI7 and I7 sites. . The contribution of LF to the total SOC stock was in average about 22.9% but more variable values were observed among the sites. In this case the highest values were found in the two 30 years old olive groves (24% in I30-A and 36% in I30-B), while the lowest contribute was measured in control (14.1%), probably due to the removal of the plants residues in cropland; intermediate values were observed in SI7 (20.8%) and I7 (19.4%).

The measures of ^{14}C activity in samples of bulk soil, HYD and REC fractions, expressed as percent in modern carbon, are reported in Figure 3.6 b. The horizontal line, at 100 pMC value, indicates the limit of modern carbon. A similar ^{14}C content was measured in bulk soils of control, SI7 and I7, with a high percentage of modern carbon (included between 95.4 and 97.9), while a depletion in ^{14}C content appeared in bulk soils of I30-B (87.1) and an enrichment in bulk soils of I30-A (101.2). The HYD fractions followed a completely different behaviour, in fact in control, I30-A and I30-B, the fractions showed a pMC value next to those of the relative bulk soil signature (control 100, I30-A 95.4 and I30-B 93.4), while, in both seven years old olive groves, the HYD fraction appeared very depleted in ^{14}C (SI7 74.5 and I7 82.9 pMC). However, in HYD fraction a trend of increase in pMC was observed passing from SI7 and I7 to I30-B and –A, up to control site. The pMC in REC appeared comparable

among control, SI7, I7 and I30-B sites and strongly depleted, with a pMC value ranging between 47.0 and 49.2), while in I30-A site the REC fraction appeared enriched in ^{14}C, with a pMC value of 83.1.

The mean annual values of C sequestration in stable SOM fractions (HYD+REC), during a period of 5 years (2014-2019), was calculated by the application of a model that simulates the organic C dynamics in soil and the result is showed in Figure 3.6 c. All the olive groves showed an higher mean annual value of C sequestration in the two stable SOM fractions considered together (between 2.0 and 3.1 kg ha^{-1} of C) compared to control (about 1.3 kg ha^{-1} of C). However, respect to control the highest rate appeared in I7 and I30-A, with also comparable values among them (3.1 and 2.9 kg ha^{-1} of C respectively).

The SOM fractionation was also performed on samples from all deep horizons of soil profiles, in order to evaluate the contribution of each fraction to total SOC stock and their possible variations in depth (Figure 3.7). The SOC stock in LF (OC_{LF}) of I30-A site (Figure 3.7 a) was significantly higher in top Ap horizon, as reported above, but it strongly decreased yet at 20 cm in depth and a similar trend appeared also in the other 30 years old olive grove (I30-B), while in all other conditions (both control and olive groves) no great variation through the profile were observed, with the only exception in SI7 where a little increase was observed at 40 cm in depth. The variation along soil profile of OC stock related to HYD fraction (OC_{HYD}) (Figure 3.7 b) followed a similar trend observed for bulk soil, in fact in a depth included between 20 and 40 cm, the OC_{HYD} increased in control, I7 and SI7, whereas in I30-A and I30-B a quick reduction of OC_{HYD} yet in the second horizon was recorded. In general no difference among I7, I30-A and control were observed in term of OC_{HYD} stock along the profile.

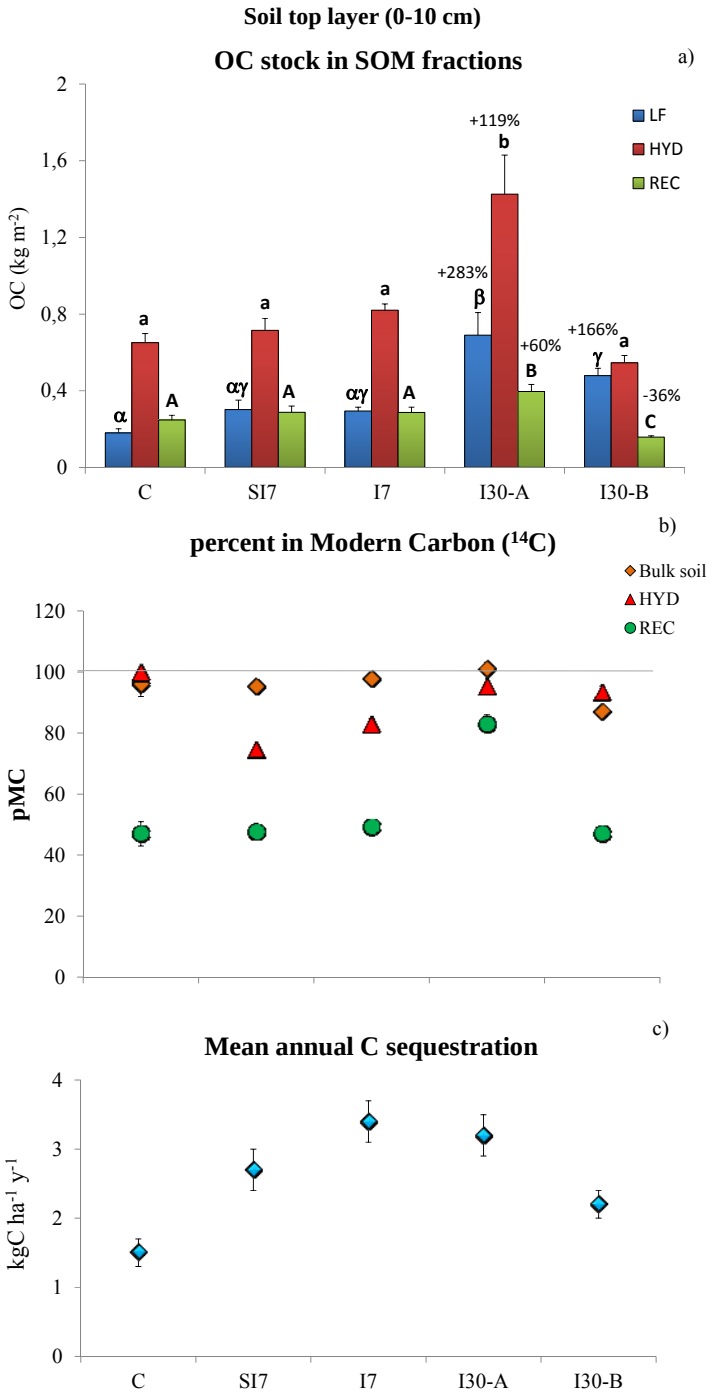


Figure 3.6 Figure reports in (a) mean values (+ standard error) of organic carbon stocks in SOM fractions (LF, HYD and REC)=; in (b) percent in Modern Carbon in bulk soil and in HYD and REC fractions (pMC value ± statistic error); in (c) mean values (± statistic error) of annual C sequestration in HYD and REC fractions calculated for the next 5 years in soil top layer (0-10 cm). Different letters on bars indicate significant differences among OC in each SOM fraction of the four experimental conditions (a specific font type was used for each SOM fraction). The percentages reported on bars indicate the relative increase (+) or decrease (-) in OC in each fraction compared to control. C=control; SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

The same estimation could be done for OC in REC fraction (OC_{REC}) (Figure 3.7 c), in this case a more clear differentiation appeared. In fact in the same depth range of the accumulation reported above the OC_{REC} stock in the control was significantly higher than all olive groves (SI7, I7 and I30-A), while the trend followed in depth by I30-A, SI7 and I7 was very similar. Therefore, clearly appeared that in control the OC in REC and HYD fractions contributed more or less equally to the accumulation in depth, probably due to the different tillage (plowing) that can influence distribution of SOC up to this depth, while in olive groves the OC was allocated in higher proportion in HYD fraction than in REC. This trend was more marked in the olive grove intensively managed 7 years old (I7) and 30 years old of Madonna de'Bagni (I30-A).
The graphs in Figure 3.8 show the summarized results for each studied site of the distribution of SOC stock in bulk soil and in SOM fractions. Data showed that in all studied soils the increase in OC in depth was associated to an increase of OC in more stable SOM fractions. In particular in control (Figure 3.8 a) the HYD and REC fractions had a similar amount of organic C, and the great amount of the stock was concentrated in the upper 30 cm, probably due to the tillage, while in the olive groves the highest proportion of the OC was allocated in HYD fraction rather than in REC fraction. Moreover, by this graph was clearly visible the lower amount of OC in bulk and SOM fraction in SI7 until about 30 cm in depth. The stocks variation along soil profile in I30-A and -B (Figure 3.8 d and e) highlighted the quick reduction, in the first soil layers, of OC content in bulk soil, associated to a decrease in the OC of SOM fractions, and the relative constant values of OC in bulk soil and fraction in the deeper soil layers. In both case (I30-A and I30-B) the REC fraction appeared strongly depleted in OC compared to HYD. However, it is necessary to underline that, despite the similar global variation in deep of the OC in bulk soil in both I30-A and I30-B sites , the cumulative amount was significantly higher in I30-A (showing also a higher content of OC in HYD and REC fractions) and this can be explained by the high carbon input in the top soil layer

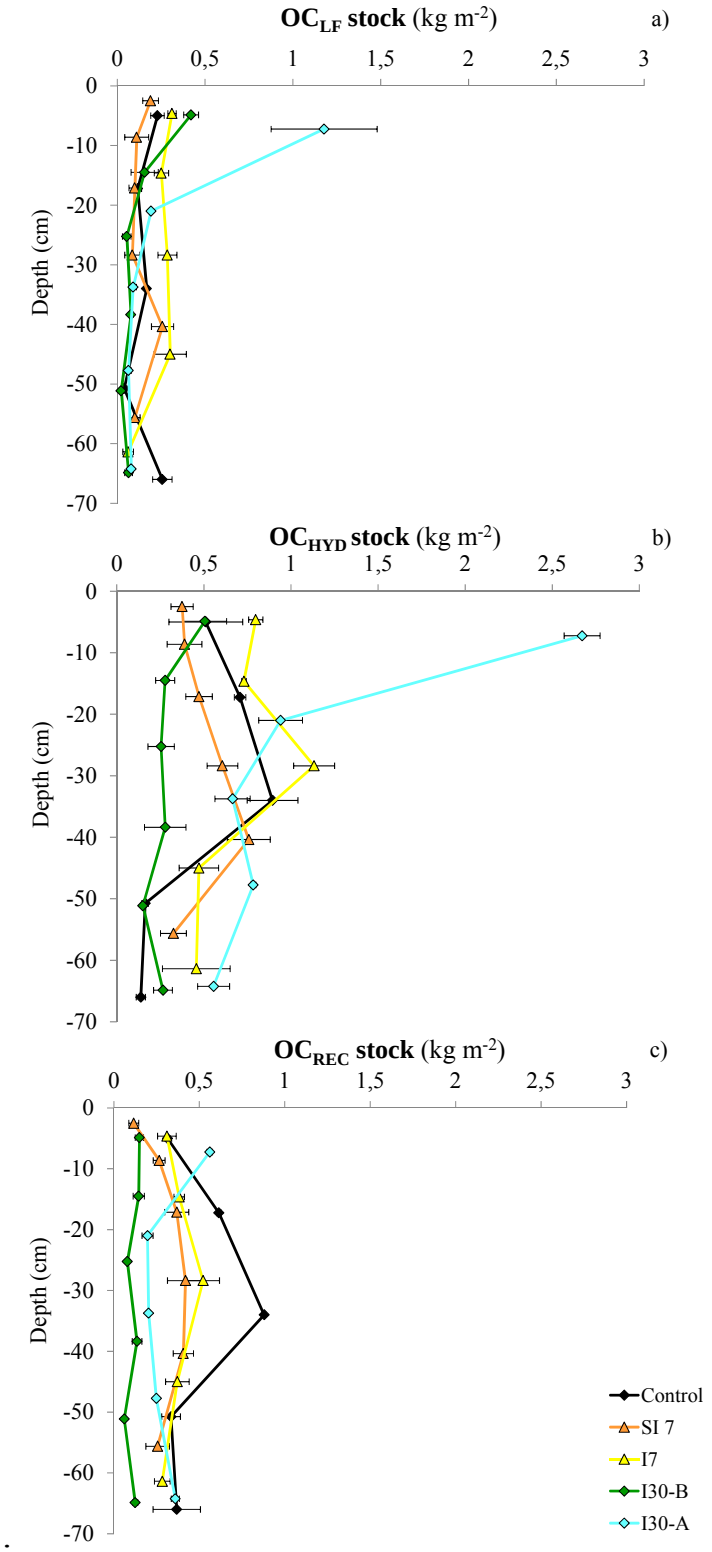


Figure 3.7 Mean values (± standard error) of variations of OC stock in the three SOM fractions along soil profile for each site. SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

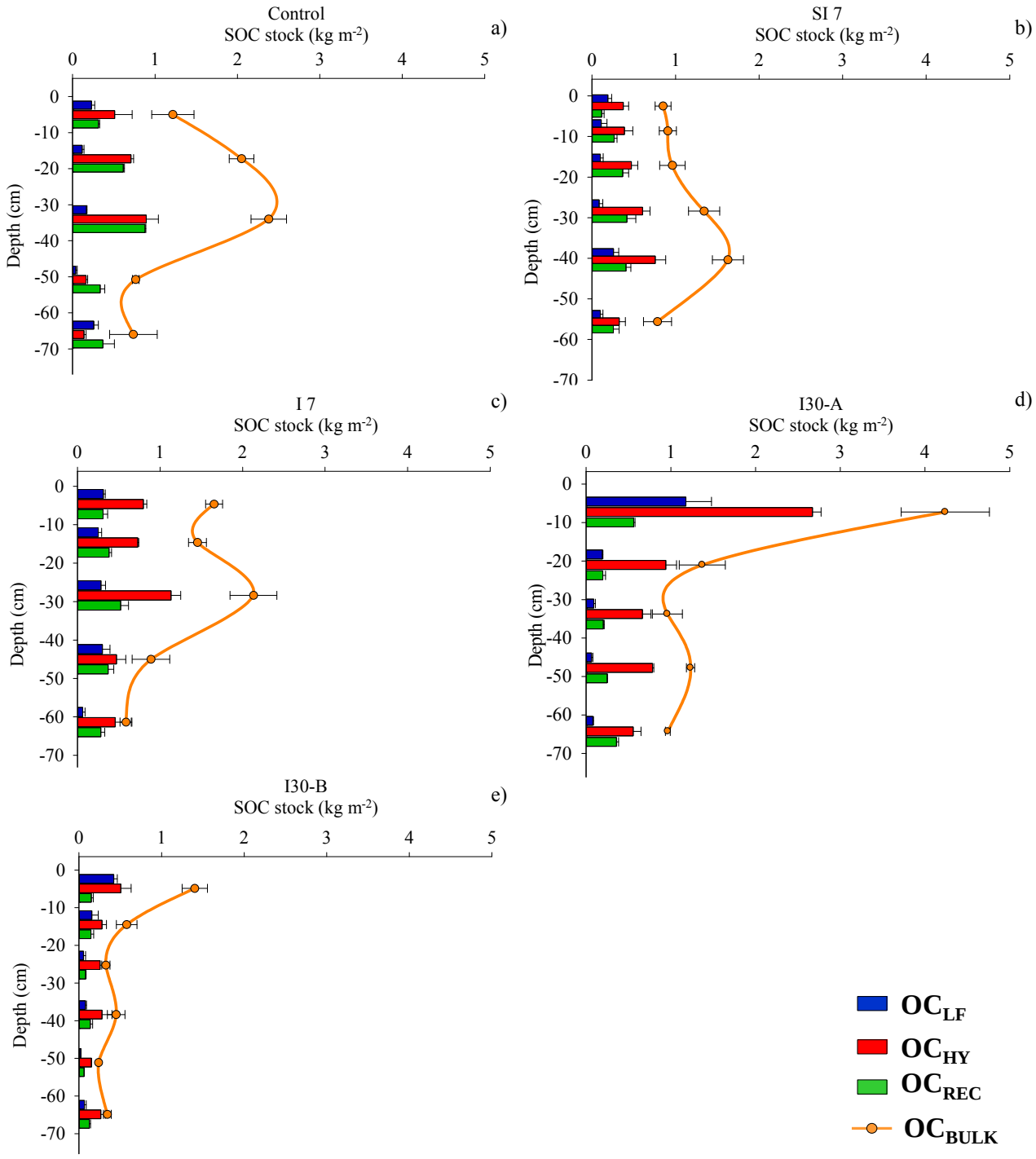


Figure 3.8 Summarized results of trend in OM within LF, HYD and REC fractions and bulk soil along profile.

The cumulative OC stock in SOM fractions (kgC m^{-2}) is represented in Figure 3.9, while in Table 3.4 it is reported the OC stock for each fraction converted in MgC ha^{-1} and the same stocks calculated at 30 cm in depth. The OC_{LF} stock measured at 30 cm of depth appeared higher in I30-A compared to all other experimental conditions; the OC stocks in HYD and REC fractions were similar among control, SI7, I7 and I30-A (Table 3.4). The cumulative OC stock measured in REC fraction of I30-A was significantly lower compared to control (on average 2.5 kg C_{REC} m^{-2} in control and 1.5 kg C_{REC} m^{-2} in I30-A), but similar to both 7 years old olive groves (SI7 and I7, that were similar also to control), and a significant lower value in OC of HYD fraction compared to I7 ($P<0.05$) (3.6 and 2.0 kg C_{HYD} m^{-2} in I7 and I30-A respectively). Comparing the two 30 years old olive groves (I30-A and I30-B) we observed differences only in the cumulative amount of OC in REC fraction, that was lower in I30-B while a comparable stock in LF and HYD fractions were measured. No difference was observed for the cumulative amount of OC in LF among all experimental conditions, however it was possible to observe a trend of increase (even if not significant) in relation to the increase of the years of plantation, due to the higher inputs of plants residues in the years and also to the management practice adopted.

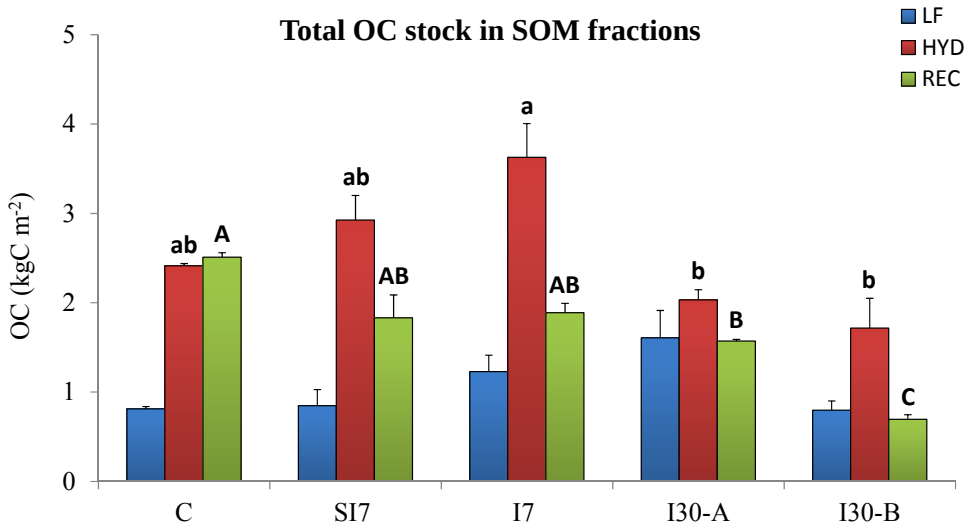


Figure 3.9 Mean values (+standard errors) of the cumulative amount along soil profile of the OC stock in SOM fractions. Different letters indicate significant differences among experimental conditions.

Table 3.4 Mean values (±standard deviation) of SOC stock (MgC ha^{-1}) in the three soil organic matter fractions calculated for the top 30 cm of soil and as total stock along soil profile. Different letters indicate significant differences between the experimental conditions.

	SOM-C stock (MgC ha^{-1}) (0-30 cm depth)			Total SOM-C stock (MgC ha^{-1}) (whole profile)		
	OC_{LF}	OC_{HYD}	OC_{REC}	OC_{LF}	OC_{HYD}	OC_{REC}
Control	4.0^{a}	15.05^{ab}	11.9^{a}	8.1	24.1^{ab}	25.1^{a}
	(±1.2)	(±0.05)	(±1.4)	(±0.4)	(±0.4)	(±0.7)
SI7	4.0^{a}	15.2^{ab}	9.5^{a}	8.5	29.3^{ab}	18.3^{ab}
	(±2.5)	(±2.3)	(±3.4)	(±3.7)	(±5.5)	(±5.1)
I7	7.3^{a}	21.2^{b}	9.7^{a}	12.3	36.3^{a}	18.9^{ab}
	(±1.5)	(±4.9)	(±2.6)	(±3.7)	(±7.5)	(±2.1)
I30-A	13.9^{b}	13.6^{ab}	7.9^{a}	16.1	20.3^{b}	15.7^{b}
	(±4.4)	(±1.4)	(±0.6)	(±4.3)	(±1.6)	(±0.3)
I30-B	6.3^{a}	10.1^{a}	3.5^{b}	8.0	17.2^{b}	6.9^{c}
	(±1.6)	(±4.2)	(±0.3)	(±2.1)	(±6.7)	(±1.0)

SI7= super-intensively, 7 years old; I7= intensively, 7 years old; I30= intensively, 30 years old (-A: in Madonna de'Bagni, -B: in Monticchio).

3.4. Discussions

Tree crop plantation, especially the perennial ones, have a large sequestration potential and are able to sequester C for longer periods with small annual fluctuations. Many annual crops such as maize can fix more C than forestry systems in any given year, but their biomass usually decomposes rapidly, and the rate and return of sequestered C to the atmosphere are very fast (Liguori et al., 2009). Perennial tree crops fix C in the permanent structures of the plants, fruits and leaves, but the C of fruits is lost from orchard system through harvesting, the C of leaves is converted in SOC when leaves are decomposed, while the C fixed in permanent structures, represent an important pools of fixed CO_2 extracted from atmosphere for a period equivalent to the life of trees. Sofo et al. (2005) estimated that in a period of 5 years olive trees were able to fix CO_2 in leaves and pruning material in an amount ranging between 3.78 and 3.62 t ha^{-1} respectively, 6.70 t ha^{-1} in olive fruits, while the humus derived from the decomposition of senescent leaves and pruning material was about 0.18 and 1.51 t ha^{-1}, respectively. In more mature system generally the amount of fixed CO_2 increase, reaching value of 7.87 t ha^{-1} in pruning material (Sofo et al., 2005). Therefore Mediterranean orchards may be important in the mitigation of CO_2 releases into the atmosphere by carbon immobilisation and humus production, particularly olive groves in Mediterranean countries where are economically important and widely cultivated (Sofo et al., 2005). Because of the SOC content depends by soil characteristics and C balance between C inputs and outputs, the land use, land use change or soil management that act on this condition, might affect the SOC stock. For example the elimination of a native vegetation substituted by a tree crop system significantly reduce the C inputs and increase the C losses due to: low density of the planting (Nieto et al., 2012); tillage that favours the mineralization and affects the physical and chemical condition of the soil (moisture, aeration, nutrient availability, physical accessibility of the organic matter by microorganisms), destruction in uppermost soil layers of soil structure with greater C loss and increase

in the rate of erosion. However in an agroecosystem, the introduction of alternative soil-management practices to conventional tillage, such as leaving the ground untilled, sowing cover crop or increase C inputs, can decrease the erosion and increase the fertility of olive groves (Castro et al., 2008; Gómez et al., 2009; Hernández et al., 2005; Rodríguez-Lizana et al., 2008). It was estimated a carbon sequestration potential of 0.3 t C ha^{-1} y^{-1} after changes in cropland use, and introduction of the best management techniques for every land use and climate zone (IPCC, 2000). However other factor that affect SOC content of agricultural soils, over management and soil properties, is climate (Lal, 1997). Therefore following these three aspects will be discussed for our experimental conditions.

As concern the climate Chiti et al. (2012) underlined, in their estimation of the SOC stock in the top 30 cm of in Italian soils under different land use, that it plays a very important role. In particular for olive groves, distributed in the most typically Mediterranean type of climates, the minimum and maximum SOC stock values were observed respectively in the lowland of the Mediterranean sub-continental to continental type of climate (42.1 MgC ha^{-1}) and in Mediterranean sub-oceanic to oceanic type of climate (56.0 MgC ha^{-1}). Umbria region, in which our olive groves are located, is included in two type of climate that they identified, temperate sub-continental to temperate continental partially mountainous and temperate sub-continental to temperate continental partially mountainous, and the SOC stock measured in our olive groves (in average 38.5 MgC ha^{-1} considering all 4 olive groves, and 42.7 MgC ha^{-1} excluding I30-B) was in agree with the mean value found in both type of climate by Chiti et al. (2012) (47.0 MgC ha^{-1} and 56.0 MgC ha^{-1} respectively for the first and second kind of climate). Also the SOC stock measured in the cropland (40 MgC ha^{-1}) agreed with those reported in this study for arable land included in the same climate type (between 46.1 and 49.5 MgC ha^{-1}). However the mean value in C stock considering all the experimental condition in a unique category of cropland (38 MgC ha^{-1}) was in average lower than the mean value of Italian

cropland (52.1 MgC ha^{-1}) (Chiti et al., 2012), and also compared to the estimation of the European agricultural area reported by Smith et al. (1997) (53 MgC ha^{-1}), but excluding the I30-B that showed a different behavior that other condition, the mean value (42 MgC ha^{-1}) appeared only slightly lower than this estimation. As underlined by Chiti et al. (2012), in the Italian estimation, the arable land and olive groves contain significantly higher mean C stock that the other subcategories considered.

Our results of OC stock (kg m^{-2}) in 0-50 cm depth in I30-A were comparable to the stock found by Agnelli et al. (2014) in a other type of tree crop in Italy (10 years old vineyard) harrowed in the same climate region, but lower than a vineyard grass-cover; moreover also the stock in SI7 and I7 were lower than those reported in this study in vineyard. In term of total N stock (kg m^{-2}) at 50 cm in depth, we found that in I30-A the stock was lower compared to those found by Agnelli et al. (2014) in harrowed vineyard, but were higher than those that they found in vineyard cover crop and in the control (a field naturally cover by grass, that in the past was a cereal cropland). Therefore the results about the soil organic carbon and total nitrogen storage in the studied experimental conditions are comparable with some literature findings in Italian and European olive groves. In 40 years old olive groves in Southern Italy under traditional tillage, Novara et al. (2012) measured in the upper 40 cm a of soil a SOC stock (53±13 MgC ha^{-1}) comparable with those measured in our experimental site, in average very similar to I30-A and I7.

Lower SOC and TN stocks were measured by Lozano-García and Parras-Alcántara (2014) in a 30 years old olive groves under conventional tillage in a typical Mediterranean climate either in top layer (0-30 cm) and total amount in whole profile compared to I30-A (SOC stock: 15.7 MgC ha^{-1} *vs* 53.7 MgC ha^{-1} in top 30 cm and 65.7 MgC ha^{-1} *vs* 87.7.4 MgC ha^{-1} in entire profile; TN stock: 2.03 MgN ha^{-1} *vs* 6.8 MgN ha^{-1} in top 30 cm and 8.5 MgN ha^{-1} *vs* 11.7 MgN ha^{-1} in entire profile) (Table 3.2) but similar in top 30 cm depth (22.1 MgC ha^{-1}, 3.0 MgN ha^{-1}) and lower in entire profile (33.3 MgC ha^{-1}, 5.4 MgN ha^{-1}) compared to I30-B; however it is necessary to

underline that I30-A showed a similar percentage of silt than those reported by Lozano-García and Parras-Alcántara (2014), while in I30-B it was lower. Therefore considering the influence of climate the OC content measured in our olive groves and control were in agree, and sometimes they appeared also richer, with other estimates in similar kind of tree crop system in Mediterranean region and Italy.

As concern the soil properties, the texture and the mineralogical composition are some of the parameters that significantly affect the C stored in the soil (Nieto et al., 2012), in particular the clay content had a direct relation with C input and accumulation, because of the presence of clay causes a low mineralization rate of SOC and release of CO_2. Consequently clayey soils need less input from plant debris to reach the same SOC value as sandy soils (Hassink, 1997) due to a physical protection exerted by clay- or silt-sized micro-aggregates on SOC against biodegradation (Balesdent et al., 2000). Simply it is possible to affirm that in soil a relation between the SOC and the clay content exists, in fact generally to a low clay concentration a low SOC content is associated, while higher concentration of clay produce a higher concentration of organic carbon in soil. This important aspects about the influence of soil characteristics on SOC accumulation explain the lower SOC content found in the top layer in I30-B compared to I30-A, that are olive groves with same age of the actual plantation and also subjected to a similar management but that differed in clay content. We found in top layer (Figure 3.2) a mean OC content of 2.8 kgC m^{-2} in I30-A with in average 26% of clay size particles, compared to 1.3 kgC m^{-2} found in the other one with a mean value of clay of 9.5%, and differences appeared also considering the 0-30 cm upper soil (Table 3.2). Probably in I30-B, supposing a comparable C input to I30-A, a C loss associated to erosive processes occurred, affecting principally the loss of fine particles (Caravaca et al., 1999; Francia Martínez et al., 2006). In fact the main C pool mobilized by erosion is the most stable form, as well as those associated with the mineral fraction, while soil management preferentially acts on the most labile fraction and the contribution of

erosion to C loss is very high for degraded soils with very low levels of SOC, as is common in semi-arid areas (Martinez-Mena et al., 2008) or generally in managed agroecosystem. Instead more similar in clay content and SOC stocks, appeared the control, SI7 and I7, while in I30-A despite a lower clay content, a significant higher SOC in top soil (0-10 cm) was measured, and it was probably due to the higher C inputs and longer period of management as tree crop system. The OC content in the upper 30 cm in I30-A was higher compared to the data reported by Nieto et al. (2012), measured in conventional tilled olive groves in Spain despite the lower percentage of clay measured in our condition (about the 28% of clay in average). While the two tilled 7 years old olive groves showed a comparable amount of SOC, between 35 and 43 MgC ha^{-1} in SI7 and I7 respectively, perfectly in agreement with the stock measured by Nieto et al. (2012) in an olive grove with a similar percentage of clay (32 MgC ha^{-1}, about 40% of clay). However, the TN stock calculated at 30 cm depth in I7 and I30-A were higher compared to those measure by Nieto et al. (2010) in olive grove under conventional tillage (3.8 tN ha^{-1}, in our case 5.2-6.8 tN ha^{-1}).

The last aspects to take in count, is the management strategy adopted in olive groves. The land use change from arable land to a tree crop system was few time investigated in term of effects on SOC and TN stock (Novara et al., 2012; Parras-Alcántara et al., 2013). For example Parras-Alcántara et al. (2013) observed that the conversion from arable crop to olive grove or vineyard caused a reduction in SOC stock between 64-52% and between 38-42% for TN stock, however our data are not comparable with those reported by these authors for the very different soil profile depth. Anyway in general they observed a reduction in depth of SOC concentration, related to a reduction in clay content in deep horizons, and the SOC stock that varied along soil profile, showed the higher value in Ap and Bt horizons (Parras-Alcántara et al., 2013). Novara et al. (2012) reported that land use change strongly affect the SOC stock, in particular they measured an increase in SOC of 105% after 30 years from the conversion from arable land to vineyard managed with conservative tillage and a

C loss of 50% when the conversion from natural condition (garrigue) to olive grove occurred; however the conversion from arable land to olive grove was not contemplated in this work. The annual routine tasks involved in olive cultivation generate a number of residues that are well suited for recycling as soil covering around the trees, such as the waste left from cleaning the fruit prior to extracting the oil (leaves, green twigs) and small branches derived from regular pruning. Traditionally, this residues were often burned resulting in a general loss of carbon and considerable CO_2 emissions (Nieto et al., 2010). Therefore cover the ground with pruning debris might help to adsorb atmospheric CO_2 and store it as organic matter in the soil (Sofo et al., 2005), so pruning material left to decompose naturally represent an efficient mean of long-term CO_2 immobilisation (Lal, 1997). In fact, 1 year after plant residues are added to the soil, most of the carbon returns to the atmosphere as CO_2, but one-fifth to one-third remains in the soil either as live biomass or as soil humus (Brady and Weil, 2004). Pruning materials and dead leaves could be used to improve the soils physical, chemical and biological properties, including greater water holding capacity and availability of plant nutrients. The management practice of mulching with shredded olive-pruning debris and residues from cleaning the fruits, is considered a practices able to increase the SOC and total N stock, however little information are available about the C storage derived from this practice (Nieto et al., 2010). For example a higher SOC stock was measured in the olive grove managed with mulching ranged between 113 and 158 tC ha^{-1} compared to 26-27 tC ha^{-1} measured in a conventional tillage olive grove (Nieto et al., 2010), with a C sequestration rate estimated around 0.5 and 0.6 t C ha^{-1} y^{-1}. Moreover also Repullo et al. (2012) reported some data that supported the effectiveness in the improvement of SOC stock after the superficial spread of olive residues, measuring an increase in SOM content in the first 5 cm of soil of 11.4 Mg ha^{-1} compared to control, when pruning residues with a size bigger or smaller of 8 cm in diameter where applied at the rate of 7.5 kg m^{-2}. The olive groves studied in our work showed lower value in

SOC stock than olive groves managed with mulching (Repullo et al., 2012) but higher than those traditionally tilled reported by Nieto et al. (2010). Probably this discrepancy is related to the fact that our olive groves, that received every year olive pruning residues, practices introduced about 10 years ago in the studied area, are evenly tilled (at a deep of about 20 cm), and this can determine a higher SOC loss compared to a no tillage strategy, moreover in our olive groves also a control of weed by disc harrow and cultivator is done about twice a year. Also the management strategy of cover crop is considered a valid strategy in improvement of SOC stock in olive groves. For example Nieto et al. (2012) observed that the change from conventional tillage to cover crop strategy in olive groves caused an increase in SOC, especially in soils more degraded by tillage and after longer time of cover use (14 years), due to increase in annual C input (C supply by the plants), reduction in C losses and in the impact of primarily erosion (Smith, 2007). However comparing our olive groves SI7, I7, I30-A, that appeared turfed because of weed are not controlled by herbicides but only by cultivator, and also control with those subjected to a cover crop management, a comparable amount of SOC at 0-30 cm depth was observed (Gómez et al., 2009; Nieto et al., 2012), and sometimes also higher, in fact the SOC stock in the top 0-10 and 0-30 cm in I30-A (in average 28 and 53 MgC ha^{-1} respectively) (Table 3.1 and 3.2) were higher than those reported by Castro et al. (2008) for a cover crop olive grove in which the plant residues were buried by disk harrow (19 and 42 MgC ha^{-1} in 10 cm and 30 cm of soil respectively), or compared to the data reported by Gómez et al. (2009) in Spanish conventional tilled olive groves and by Hernández et al. (2005) either in olive grove under conventional tillage and cover crop. Therefore from the literature comparison, appeared that I30-A olive grove had a SOC content comparable or higher than olive groves in cover crop condition, suggesting that it was probably due to the time of management with tree crop and also the practice of mulching with pruning residues, that could be considered a conservative management practice. The literature finding actual available, does not

support a clear and exhaustive interpretation of our observation, because of the change from a crop system to other one, such as cropland to tree crop, are under investigated compared to land use change from a natural condition to a managed one, more studied because of the negative effects on SOC storage and consequently increase in CO_2 emission are known.

In natural ecosystem as well as in agroecosystem, an important component of the SOC stock is the carbon stored in deeper horizon. In fact even if most of the literature studies about the SOC stock and its response to climate change and land use changes have focused on the upper 20-30 cm of soil, where the higher C concentration and greater microbial activity are usually found, only few include the total soil profile (Albaladejo et al., 2013; Koarashi et al., 2012). More than 50 % of the total SOC is stored below 20 cm depth (Batjes, 2014; Jobbágy & Jackson, 2000). The paucity of data about the soil carbon distribution in the profile in different landscapes, represent an important knowledge gaps in soil science (Kimble et al., 1998). The subsoil stocks of organic C was traditionally considered subjected to a minor shift compared to top horizons, but recent evidence had highlight that a fraction of the C subsurface is dynamic, and the C cycling associated to this pool can be significant at the global scale (Koarashi et al., 2012) with a considerable impact on the entire C balance (Don et al., 2008). To this, it contributes also the action in depth of controlling factors on C dynamic that are different from those acting in topsoil (Salomé et al., 2010). Moreover, to investigate about the impact of the key factors controlling the levels of organic matter in soils, in order to identify optimal strategies for land management (Chaplot et al., 2010), is a very important goal, being the soil C sequestration an important component of greenhouse management strategy for climate change mitigation (Pacala & Socolow, 2004). Generally going down along a soil profile, the OC concentration in bulk soil tends to decrease, whit often significant variation compared to the uppermost layer (~20 cm) related also to land use; for example in the top 40 cm of forest soils significant higher value in OC concentration may be found

than shrublands and croplands (Albaladejo et al., 2013). This suggest that the conversion from forestland or shrubland to cropland implies a reduction in SOC and the reduction is higher in upper layer and decreases in intensity with soil depth, so that below 40 cm generally no differences in SOC in relation to land use are found (Albaladejo et al., 2013). Considering the SOC concentration (expressed in gC kg^{-1}, Figure 3.2) a similar behavior was observed in our experimental condition compared to those reported by Albaladejo et al. (2013), in fact they found that the SOC for all land use decreased significantly with depth, while in term of mean SOC stock in the entire soil profile, that amounted to 76 MgC ha^{-1} in average (excluding I30-B), was slightly higher than the mean SOC stock reported in this study (63 MgC ha^{-1}) for the general category of cropland (including irrigated cropland as cereals, fruit trees and citrus, and also in small proportion not irrigated as cereals and almonds).

In general, the deep trend in I7, SI7 and I30-A of SOC concentration was very similar to those observed in other type of Italian tree crop system, such as vineyard, by Agnelli et al. (2014) with a gradual reduction in OC concentration along soil profile, however in this study the authors underlined a lower OC concentration in top 20 cm in harrowed vineyard compared to cover crop vineyard and control, condition not observed in our case, because of I30-A in Ap horizon was significantly higher that control and in other olive groves the superficial concentration was comparable but not lower than the control. Moreover more in depth, Agnelli et al. (2014) measured a higher OC concentration in vineyard than control, while in our soils the control and both 7 years old olive groves followed a similar trend and showed a comparable concentration, and only I30-A at about 30 cm in depth had a lower concentration than control and I7. Similarly to our results, also Syswerda et al. (2011) did not observed consistent differences in soil C at depth comparing annual cropland and perennial tree plantation of poplar. The control factors, their relative importance and the mechanisms involved in the stabilization and dynamics of SOC may be different at the surface and in subsurface soils (Rumpel & Kögel-Knabner, 2010) and also may

change in depending on land use. In cropland, as well as in shrubland, temperature and lithology were the main control factors in the variability of SOC concentration through the soil profile, but whereas, the relative importance of climatic factors (precipitation and temperature) decreased with depth, the importance of textural factors (clay and fine silt) and lithology increased, that determine the stabilization of organic matter through organo-mineral association in deeper layers (Six et al., 2002). In addition the potential capacity for additional C sequestration could be higher in cropland soils due to the lower C saturation (Álvaro-Fuentes et al., 2012). Therefore, the application of the soil organic matter fractionation could be a useful tool in order to better understand the main factors and processes that can determine the C sequestration in a studied soil. In particular the fractionation procedure that we applied allowed firstly the separation of mineral-free LF, than of a chemically labile mineral-associated OM fraction (called hydrolysable) and at the end a chemically recalcitrant mineral-associated OM (non-hydrolysable), namely pools that turnover on different time scale and identifiable as active, intermediate and passive pool respectively. The chemical fractionation causes the extraction of mineral associated organic matter, by the H^+-bridges, polyvalent cation bridges, hydrolytic bounds, complexes with Fe and Al. Each separated fraction had a different ecological means, and a different response to management. This method was more wildly applied in more natural environments such as forest or grassland and less in agricultural ecosystem. Low-density carbon (LF) is commonly considered a fast-cycling carbon pool in soils, consisting in relatively fresh plant material not associated with minerals, very variable in space and time and it is strongly dependent by C inputs level and quality, being constituted by young detritus and fine roots (Wäldchen et al., 2013). It forms generally a most abundant pool in superficial horizons, however in depth it could be equally a large fraction of the OC especially in coarse textured subsoils (Koarashi et al., 2012) where the LF is physically protected by aggregates formation that make organic materials less accessible for soil microbes and enzymes (Baisden et

al., 2002; Rasmussen et al., 2005; Six et al., 2004), this cause an increases in ^{14}C-LF age but the chemical structures is similar compared to LF in surface layers. Generally LF of SOC are known to respond faster to land-use and management changes than the more stable fractions (HYD and REC) (Hassink et al., 1997; Leifeld & Kögel-Knabner, 2005). For example Meyer et al. (2012) observed that, after a soil abandonment, the particulate organic matter, similarly a labile SOM fraction, was the faction most affected by land use change, while occluded particulate organic matter and mineral associated organic matter showed only a slight or no accumulation of C, even if in theme was included the major part of SOC. However if a marked effect on LF is expected in land use change from natural to managed condition or in the opposite variation, in our study in which we take in count the conversion from a cropland to a more conservative agroecosystem, it may be not evident. In fact we observed that the OC pool in LF was significantly improved by the olive grove only after a longer period and only in top layer, in fact it contribute in a great percentage to SOC stock in both 30 years old olive groves (24% in I30-A and 36% in I30-B). In both 30 years old olive grove the OC_{LF} stock gradually reduced in depth and no great differences were observed among all sites, but observing the Figure 3.7, it is possible to affirm that, in depth, in control, SI7 (a) and also I7 (b) this pool contributed more to the global C stock. The presence in depth of this faction was due in agricultural ecosystem to the plough that allow the distribution of this material until to 20-30 cm, and more in depth the great contribution was probably related to the roots debris inputs. The LF could be stabilized in soil aggregates and accumulate in depth, where microbial activity is less pronounced, causing the accumulation of this fraction and its contribution to the global stock. This effect was more marked in olive groves (especially I7 than SI7, I30-A and -B) compared to control where inputs of plant residues is lower. The influence of land use change of LF was also reported by Ramnarine et al. (2015) affirming that in conventional tillage the LF is reduced for the aggregate disruption, and they found after 6 years of no tillage a significantly

higher amount only in top layer of soil (0-10 cm) (5.8 gLF kg^{-1} soil) than conventional tillage (4.2 gLF kg^{-1} soil) (+37%), contributing for 6.8% to total OC. However in our study, we can relate the increase in LF principally to land use and to the tree pruning residues left in field because of the olive groves are ploughed, but the increase appeared only after several time (30 years or more). However some literature finding put in evidence that not only the more labile form of organic matter but also the more stable ones could be affected by the soil management. McLauchlan et al. (2006) found that in a chronosequence of conversion from cropland to perennial grassland over a period of 40 year, the total soil OC increased in the top 10 cm of soil with an increase in both labile and recalcitrant organic carbon pools (measured as unhydrolyzed C), hypothesizing that a large proportion of new C inputs from plants quickly became stabilized in the soil, and formed a stable OC pool in few decades of grassland establishment. Similarly, Valboa et al. (2015) found in maize cropland under minimum tillage an increase in labile organic carbon up to 30 cm in depth, and in a management of ripper subsoiling, an increase in recalcitrant fraction in top 10 cm of soil after 17 years from the adoption, while in conventional tillage loss of REC OC was found. These observations are in agree with the increase in OC in both HYD and REC fractions found in I30-A in top layer that was significantly higher compared to control and both 7 years old olive groves. In particular the increase in OC_{HYD} and OC_{REC} compared to control was respectively of 119% and 60%. However we found that in cropland the OC_{REC} in proportion contributed more to the top layer OC stock than in olive groves, and the data were in agree with those reported by Benbi et al. (2015) in the comparison among different agricultural ecosystems and uncultivated sites, they found that agroforestry (poplar plantation) had a lower proportion of recalcitrant C (23-26%) than maize-wheat (37%) or rice-wheat (42%) cropping systems. Both HYD and REC fractions are stabilized in soil by mineral interaction and their chemical properties and both this factors determine the C dynamic in soil and the permanence time of the OC in each fractions. The HYD fraction is considered

the most important ecological fraction for its intermediate turnover time, able to respond on the time scale of anthropogenic global change (Townsend et al., 1995), while the REC fraction is the most important component of the permanent C sink, for its high MRT. Therefore despite the increase in OC in REC fraction compared to control was lower than those observed for HYD faction, it was equally important because of its increase, probably due to biochemical resistance of organic C molecules contained either in plant material (Kögel-Knabner, 2002) or metabolic byproducts derived from microbial metabolism (Kiem & Kögel-Knabner, 2003) and further strong stabilized in soil, supporting literature finding about the improvement of OC in REC fraction in a relatively short period, as well as the time of a management strategy adoption. The effect of the improvement of OC stock in HYD and REC fraction due to three crop management is highlight also by the enrichment in ^{14}C that cause an increase in pMC value in these fractions in I30-A. In this site in fact the surprising enrichment of the REC fraction in modern C, indicate that soil OC derived from the more recent history in term of land use has been stabilized in the SOM fraction identifiable as the soil passive pool, confirming the increase in OC recorded in REC fraction in I30-A compared to other olive groves and control. The all other experimental conditions, showed a strong depletion in ^{14}C in this fraction, with a consequent higher age of the C, indicating that the passive pools in these conditions did not received any inputs during a relative recent period. The increase in OC_{HYD} fraction in I30-A was coupled with the increase in pMC compared to the two younger olive groves. Also crop land and I30-B appeared very enriched in ^{14}C in HYD fraction, but not followed by a significant increase in OC content of this fraction. It suggests that the prolonged management of olive grove really improves the OC stocks in both intermediate and passive pools (HYD and REC fractions), acting positively on the soil potential C sequestration in these specific conditions as also underline by higher mean annual C sequestration obtained for the stable fractions

in all the olive groves compared to control, with little more higher value in I7 and I30-A (3.1 and 2.9 kgC ha^{-1} respectively) that appeared the more conservative sites.

As concern the C dynamics, its size and properties along soil profile and in subsurface horizons for both OC in bulk soil and SOM fractions, the actual understanding are still extremely fragmentary (Rumpel & Kögel-Knabner, 2010). The quantification and the study of the mechanisms of stabilizing C in deep soils such as appropriate options of land management able to increase SOM stocks (Lal, 2002; Smith, 2007) are necessary, because of it has been estimated that, increasing SOM concentrations in deep soils by 5-15% could decrease atmospheric CO_2 concentrations by 16-30% (Baldock, 2007; Kell, 2011). However the estimation of SOC stock at the global or even regional scale and detect its changes, is difficult for the natural temporal and spatial variability in soils, for the relative paucity of data on the variations in soil depth and distribution of C with depth (Dungait et al., 2012). Moreover an additional difficulty may be the accurate assessments of SOM change in determining a very small difference between two very large values. The stability of organic carbon increases with soil depth, reflecting the fundamental differences in the mechanisms of soil organic matter (SOM) stabilization in deep horizons where the relative amount of mineral associated C (high density fraction) increases compared to C relatively free of mineral particles (low density fraction) (Rasmussen et al., 2005; Six et al., 2002). The main C source of OM in subsoil are dissolved organic carbon (Kaiser & Guggenberger, 2000), root biomass (Rasse et al., 2005), physically and biologically transported particulate organic matter (Don et al., 2008), increasing the proportion of the more stable compounds. The deeper SOM is assumed to be very stable, with radiocarbon ages of more than 4000 years (Jenkinson & Coleman, 2008). In depth among all the factors that determine the OM stabilization, the physical protection by the occlusion within aggregates (Six et al., 2000), the sorption of the OC on clay minerals and amorphous iron and aluminum colloids play the major role (Kiem & Kögel-Knabner, 2003; Six et al., 2002), so organo-mineral complexes are

likely to be the primary mechanism whereby SOM is stored for millennia ("passive pool"). This stabilization mechanism is very important in depth as demonstrate by the correlation between the vertical OC distribution and the clay content found by Jobbágy and Jackson (2000). Therefore the formation of soil aggregated due to the presence of clay and silt fractions enhances the protection because a combination of adsorption and occlusion operate together (Martens et al., 2004). These important information allow as to make some considerations about the variation in depth of the HYD and REC fractions (Figure 3.6) that were the OC pools that contributed in huge quantity to whole SOC stock. In I30-A the high amount of OC in both these fractions in surface was related to the tree crop system (for more than 30 years) because of, despite the lower percentage of clay compared other condition, in top layer a significant C sequestration occurred; while in depth the reduction was less influence by the tree crop and more to the reduction in percentage of silt a clay compared to top horizon and also to other sites (control, SI7 and I7), effect more marked for REC fraction than HYD. Instead a completely different behavior appeared in I30-B, evident sign of the importance of silt and clay particles in the stabilization of OC. In fact as proposed by some authors (Hassink, 1997; Six et al., 2002; Wiseman & Püttmann, 2005) the preservation of new added C to the soil is affect by the degree of saturation of the protective sites in soil. Consequently, there should be a greater potential for OM preservation in the subsoil where the OM saturation of mineral particles is small. In this soil very poor in silt and clay, that with their high charged surface constituent the most important sites of bound and adsorption or protection of OM, the minerals phase was probably more saturated, and those associated to an enhanced water derange (in relation to soil texture), determine a higher C loss than C retention. This phenomenon clearly influenced the contribution of OC_{REC} to whole SOC stock in I30-B that was significantly lower compared to I30-A, and in I30-A where both whole OC stocks in HYD and REC were significant lower compared to control (Figure 3.8). As concern the comparison between control and younger olive

groves, considering that the sites (control, SI7 and I7) are very adjoining in the study area, that both 7 years old olive groves were planted on cereal cropland (as the actual condition of the control) and that all the soils had a similar texture and clay content both in surface and subsurface horizons, the significant decrease in OC_{HYD} and OC_{REC} in SI7 at about 15 and 30 cm compared to control and I7, could be an effect of the super-intensively management (characterised by a high density of plants, 2222 trees ha^{-1}), that has produced in this little period a significant decrease in OC related to this fraction, probably as consequence of the tree plantation. While more in depth, from about 45 cm, a higher amount of OC in HYD fraction in all olive groves compared to control was found.

3.5. Conclusions

The olive grove is a kind of agroecosystem able to improve soil organic C and total N stock in soil top layer in long period of management (>30 years). Both OC and TN concentrations and stocks were significantly higher in soil top layer of tree crop system, with an actual olive plantation 30 years old, but with a olive grove land use since a longer period. In shorter period, no differences between the control cropland and younger olive groves were observed. In top layer of I30-A significantly increased compared to control the organic C stock in all three fractions separated, with an higher percentage for light fraction, followed by hydrolysate fraction and the lowest percentage in recalcitrant fraction. However the data highlight that not only the OC stock in more labile fraction is improved with a land use change from crop land to tree crop, as expected, but also the OC stock in more stable fraction was improved in a relative brief period, confirmed by the enrichment in radiocarbon content measured in these fractions in top layer, underlining the potential formation in this agroecosystem of C sink that last for a long period. Moreover the mean annual C sequestration in the next 5 years in soil top layer in all olive groves appeared higher compared to control, with the highest values estimated for I30-A and I7. No great

variation in deep horizons were observed clearly related to tree crop system, because of in depth the soil texture and mineral phase composition affect the C storage more than the land use. However a potential negative on OC stock effect was observed for the super-intensive management strategy, in fact in SI7 a significant reduction in OC HYD occurred compared to control and I7. Further study are necessary to improve scientific knowledge about the land use change from crop to tree crop system, to confirm their real utilization as strategy in climate change mitigation; however our results suggest that the intensive management coupled to the superficial underground of pruning residues, are sustainable management strategy in Italian olive groves able to increase in time the soil organic C stock and improve their C sequestration, acquiring a real importance climate change mitigation strategy.

References

Agnelli, A., Bol, R., Trumbore, S.E., Dixon, L., Cocco, S., Corti, G. 2014. Carbon and nitrogen in soil and vine roots in harrowed and grass-covered vineyards. *Agriculture, Ecosystems & Environment*, **193**, 70-82.

Albaladejo, J., Ortiz, R., Garcia-Franco, N., Navarro, A.R., Almagro, M., Pintado, J.G., Martínez-Mena, M. 2013. Land use and climate change impacts on soil organic carbon stocks in semi-arid Spain. *Journal of Soils and Sediments*, **13**(2), 265-277.

Álvaro-Fuentes, J., Easter, M., Paustian, K. 2012. Climate change effects on organic carbon storage in agricultural soils of northeastern Spain. *Agriculture, Ecosystems & Environment*, **155**, 87-94.

Baisden, W.T., Amundson, R., Cook, A.C., Brenner, D.L. 2002. Turnover and storage of C and N in five density fractions from California annual grassland surface soils. *Global Biogeochemical Cycles*, **16**(4), 64-1-64-16.

Baldock, J. 2007. Composition and cycling of organic C in soil. In: Nutrient Cycling in Terrestrial Ecosystems (eds Marschner P, Rengel Z), pp. 1–35. Springer, Berlin, Heidelberg.

Balesdent, J., Chenu, C., Balabane, M. 2000. Relationship of soil organic matter dynamics to physical protection and tillage. *Soil and Tillage Research*, **53**(3–4), 215-230.

Batjes, N.H. 2014. Batjes, N. H. 1996. Total carbon and nitrogen in the soils of the world. European Journal of Soil Science, 47, 151–163. Reflections by N.H. Batjes. *European Journal of Soil Science*, **65**(1), 2-3.

Brady, N.C., Weil, R.R., 2004. Elements of the Nature and Properties of Soils, second ed. Pearson Prentice Hall, NJ.

Caravaca, F., Lax, A., Albaladejo, J. 1999. Organic matter, nutrient contents and cation exchange capacity in fine fractions from semiarid calcareous soils. *Geoderma*, **93**(3–4), 161-176.

Carlisle, E.A., Steenwerth, K.L., Smart, D.R. 2006. Effects of land use on soil respiration: conversion of oak woodlands to vineyards. *Journal of Environmental Quality*, **35**(4), 1396-404.

Castanha, C., Trumbore, S., Amundson, R. 2008. Methods of Separating Soil Carbon Pools Affect the Chemistry and Turnover Time of Isolated Fractions. *Radiocarbon*, **50**(1), 83-97.

Castro, J., Fernández-Ondoño, E., Rodríguez, C., Lallena, A.M., Sierra, M., Aguilar, J. 2008. Effects of different olive-grove management systems on the organic carbon and nitrogen content of the soil in Jaén (Spain). *Soil and Tillage Research*, **98**(1), 56-67.

Caughey, M.E., Barcelona, M.J., Powell, R.M., Cahill, R.A., Grøn, C., Lawrenz, D., Meschi, P.L. 1995. Interlaboratory study of a method for determining nonvolatile organic carbon in aquifer materials. *Environmental Geology*, **26**(4), 211-219.

Chaplot, V., Bouahom, B., Valentin, C. 2010. Soil organic carbon stocks in Laos: spatial variations and controlling factors. *Global Change Biology*, **16**(4), 1380-1393.

Chiti, T., Gardin, L., Perugini, L., Quaratino, R., Vaccari, F.P., Miglietta, F., Valentini, R. 2012. Soil organic carbon stock assessment for the different cropland land uses in Italy. *Biology and fertility of soils*, **48**(1), 9-17.

Don, A., Steinberg, B., Schöning, I., Pritsch, K., Joschko, M., Gleixner, G., Schulze, E.-D. 2008. Organic carbon sequestration in earthworm burrows. *Soil Biology and Biochemistry*, **40**(7), 1803-1812.

Dungait, J.A.J., Hopkins, D.W., Gregory, A.S., Whitmore, A.P. 2012. Soil organic matter turnover is governed by accessibility not recalcitrance. *Global Change Biology*, **18**(6), 1781-1796.

Eurostat, 2008. Agricultural Statistics. Main Results – 2006–2007. Office for Official Publications of the European Communities, Luxemburg.

Fantappiè, M., L'Abate, G., Costantini, E. 2010. Factors influencing soil organic carbon stock variations in Italy during the last three decades. In: *Land degradation and desertification: assessment, mitigation and remediation*, Springer, pp. 435-465.

Francia Martínez, J.R., Durán Zuazo, V.H., Martínez Raya, A. 2006. Environmental impact from mountainous olive orchards under different soil-management systems (SE Spain). *Science of The Total Environment*, **358**(1-3), 46-60.

Freibauer, A., Rounsevell, M.D., Smith, P., Verhagen, J. 2004. Carbon sequestration in the agricultural soils of Europe. *Geoderma*, **122**(1), 1-23.

Gardi, C., Sconosciuto, F. 2007. Evaluation of carbon stock variation in Northern Italian soils over the last 70 years. *Sustainability Science*, **2**(2), 237-243.

Gómez, J.A., Guzmán, M.G., Giráldez, J.V., Fereres, E. 2009. The influence of cover crops and tillage on water and sediment yield, and on nutrient, and organic matter losses in an olive orchard on a sandy loam soil. *Soil and Tillage Research*, **106**(1), 137-144.

Harris, D., Horwáth, W.R., van Kessel, C. 2001. Acid fumigation of soils to remove carbonates prior to total organic carbon or CARBON-13 isotopic analysis. *Soil Science Society of America Journal*, **65**(6).

Hassink, J. 1997. The capacity of soils to preserve organic C and N by their association with clay and silt particles. *Plant and soil*, **191**(1), 77-87.

Hassink, J., Whitmore, A.P., Kubát, J. 1997. Size and density fractionation of soil organic matter and the physical capacity of soils to protect organic matter. *European Journal of Agronomy*, **7**(1–3), 189-199.

Hernández, A.J., Lacasta, C., Pastor, J. 2005. Effects of different management practices on soil conservation and soil water in a rainfed olive orchard. *Agricultural Water Management*, **77**(1–3), 232-248.

IPCC 2000. Special report on land use, land use change, and forestry. Cambridge University Press, Cambridge, UK.

IPCC 2001. Climate change: the scientific basis. Cambridge University Press, Cambridge, UK.

IPCC 2007. IPCC Climate Change: The Physical Science Basis (eds Solomon S et al.), Cambridge University Press, Cambridge.

Janssens, I.A., Freibauer, A., Ciais, P., Smith, P., Nabuurs, G.-J., Folberth, G., Schlamadinger, B., Hutjes, R.W., Ceulemans, R., Schulze, E.-D. 2003. Europe's terrestrial biosphere absorbs 7 to 12% of European anthropogenic CO2 emissions. *Science*, **300**(5625), 1538-1542.

Jenkinson, D.S., Coleman, K. 2008. The turnover of organic carbon in subsoils. Part 2. Modelling carbon turnover. *European Journal of Soil Science*, **59**(2), 400-413.

Jobbágy, E.G., Jackson, R.B. 2000. The vertical distribution of soil organic carbon and its relation to climate and vegetation. *Ecological Applications*, **10**(2), 423-436.

Kaiser, K., Guggenberger, G. 2000. The role of DOM sorption to mineral surfaces in the preservation of organic matter in soils. *Organic Geochemistry*, **31**(7), 711-725.

Kell, D.B. 2011. Breeding crop plants with deep roots: their role in sustainable carbon nutrient and water sequestration. Annals of Botany, 108, 407–418.

Kiem, R., Kögel-Knabner, I. 2003. Contribution of lignin and polysaccharides to the refractory carbon pool in C-depleted arable soils. *Soil Biology and Biochemistry*, **35**(1), 101-118.

Kimble, J.M., Follett, R.F., Cole, C.V. 1998. The Potential of U.S. Cropland to Sequester Carbon and Mitigate the Greenhouse Effect. Taylor & Francis.

Koarashi, J., Hockaday, W.C., Masiello, C.A., Trumbore, S.E. 2012. Dynamics of decadally cycling carbon in subsurface soils. *Journal of Geophysical Research: Biogeosciences*, **117**(G3), n/a-n/a.

Kögel-Knabner, I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology and Biochemistry*, **34**(2), 139-162.

Lal, R. 2002. The potential of soils of the tropics to sequester carbon and mitigate the greenhouse effect. in: *Advances in Agronomy*, Vol. Volume 76, Academic Press, pp. 1-30.

Lal, R. 1997. Residue management, conservation tillage and soil restoration for mitigating greenhouse effect by CO2-enrichment. *Soil and Tillage Research*, **43**(1–2), 81-107.

Lal, R. 2011. Sequestering carbon in soils of agro-ecosystems. *Food Policy*, **36,** Supplement 1, S33-S39.

Lal, R. 2004. Soil carbon sequestration to mitigate climate change. *Geoderma*, **123**(1–2), 1-22.

Lal, R. 1999. Soil management and restoration for C sequestration to mitigate the accelerated greenhouse effect. *Progress in Environmental Science*, **1**(4), 307-326.

Leifeld, J., Kögel-Knabner, I. 2005. Soil organic matter fractions as early indicators for carbon stock changes under different land-use? *Geoderma*, **124**(1–2), 143-155.

Liguori, G., Gugliazza, G., Inglese, P., 2009. Evaluating carbon fluxes in orange orchards in relation to planting density. *The Journal of Agricultural Science* **147**(6), 637-645.

Lozano-García, B., Parras-Alcántara, L. 2014. Variation in soil organic carbon and nitrogen stocks along a toposequence in a traditional mediterranean olive grove. *Land Degradation & Development*, **25**(3), 297-304.

Martens, D.A., Reedy, T.E., Lewis, D.T. 2004. Soil organic carbon content and composition of 130-year crop, pasture and forest land-use managements. *Global Change Biology*, **10**(1), 65-78.

Martin, M.P., Wattenbach, M., Smith, P., Meersmans, J., Jolivet, C., Boulonne, L., Arrouays, D. 2011. Spatial distribution of soil organic carbon stocks in France. *Biogeosciences*, **8**(5), 1053-1065.

Martinez-Mena, M., Lopez, J., Almagro, M., Boix-Fayos, C., Albaladejo, J. 2008. Effect of water erosion and cultivation on the soil carbon stock in a semiarid area of South-East Spain. *Soil and Tillage Research*, **99**(1), 119-129.

Marzaioli, F., Borriello, G., Passariello, I., Lubritto, C., De Cesare, N., Onofrio, A., Terrasi, F. 2008. Zinc Reduction as an Alternative Method for AMS Radiocarbon Dating: Process Optimization at CIRCE. *Radiocarbon*, **50** (1), 139-149.

Marzaioli, F., Lubritto, C., Del Galdo, I., D'Onofrio, A., Cotrufo, M.F., Terrasi, F., 2010. Comparison of different soil organic matter fractionation methodologies: evidences from ultrasensitive 14C measurements. *Nuclear Instruments and Methods in Physics Research B*, **268**, 1062-1066.

McLauchlan, K.K., Hobbie, S.E., Post, W.M. 2006. Conversion from agriculture to grassland builds soil organic matter on decadal timescales. *Ecoogical Applications*, **16**(1), 143-53.

Meyer, S., Leifeld, J., Bahn, M., Fuhrer, J. 2012. Free and protected soil organic carbon dynamics respond differently to abandonment of mountain grassland. *Biogeosciences*, **9**(2), 853-865.

Morari, F., Lugato, E., Berti, A., Giardini, L. 2006. Long-term effects of recommended management practices on soil carbon changes and sequestration in north-eastern Italy. *Soil Use and Management*, **22**(1), 71-81.

Nieto, O.M., Castro, J., Fernández-Ondoño, E. 2012. Conventional tillage versus cover crops in relation to carbon fixation in Mediterranean olive cultivation. *Plant and soil*, **365**(1), 321-335.

Nieto, O.M., Castro, J., Fernández, E., Smith, P. 2010. Simulation of soil organic carbon stocks in a Mediterranean olive grove under different soil-management systems using the RothC model. *Soil Use and Management*, **26**(2), 118-125.

Novara, A., La Mantia, T., Barbera, V., Gristina, L. 2012. Paired-site approach for studying soil organic carbon dynamics in a Mediterranean semiarid environment. *CATENA*, **89**(1), 1-7.

Pacala, S., Socolow, R. 2004. Stabilization Wedges: Solving the Climate Problem for the Next 50 Years with Current Technologies. *Science*, **305**(5686), 968-972.

Parras-Alcántara, L., Martín-Carrillo, M., Lozano-García, B. 2013. Impacts of land use change in soil carbon and nitrogen in a Mediterranean agricultural area (Southern Spain). *Solid Earth*, **4**(1), 167-177.

Ramnarine, R., Voroney, R.P., Wagner-Riddle, C., Dunfield, K.E. 2015. Conventional and No-Tillage Effects on the Distribution of Crop Residues and Light Fraction Organic Matter. *Soil Science Society of America Journal*, **79**(1).

Rasmussen, C., Torn, M.S., Southard, R.J. 2005. Mineral Assemblage and Aggregates Control Carbon Dynamics in a California Conifer Forest. *Soil Science Society of America Journal*, **69**(6).

Rasse, D.P., Rumpel, C., Dignac, M.-F. 2005. Is soil carbon mostly root carbon? Mechanisms for a specific stabilisation. *Plant and soil*, **269**(1-2), 341-356.

Repullo, M.A., Carbonell, R., Hidalgo, J., Rodríguez-Lizana, A., Ordóñez, R. 2012. Using olive pruning residues to cover soil and improve fertility. *Soil and Tillage Research*, **124**, 36-46.

Rodríguez-Lizana, A., Espejo-Pérez, A.J., González-Fernández, P., Ordóñez-Fernández, R. 2008. Pruning Residues as an Alternative to Traditional Tillage to Reduce Erosion and Pollutant Dispersion in Olive Groves. *Water, Air, and Soil Pollution*, **193**(1), 165-173.

Rumpel, C., Kögel-Knabner, I. 2010. Deep soil organic matter—a key but poorly understood component of terrestrial C cycle. *Plant and soil*, **338**(1), 143-158.

Salomé, C., Nunan, N., Pouteau, V., Lerch, T.Z., Chenu, C. 2010. Carbon dynamics in topsoil and in subsoil may be controlled by different regulatory mechanisms. *Global Change Biology*, **16**(1), 416-426.

Six, J., Bossuyt, H., Degryze, S., Denef, K. 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. *Soil and Tillage Research*, **79**(1), 7-31.

Six, J., Conant, R., Paul, E.A., Paustian, K. 2002. Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and soil*, **241**(2), 155-176.

Six, J., Elliott, E., Paustian, K. 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. *Soil Biology and Biochemistry*, **32**(14), 2099-2103.

Smith, P. 2007. Land use change and soil organic carbon dynamics. *Nutrient Cycling in Agroecosystems*, **81**(2), 169-178.

Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., Howden, M., McAllister, T., Pan, G., Romanenkov, V., Schneider, U., Towprayoon, S. 2007. Policy and technological constraints to implementation of greenhouse gas mitigation options in agriculture. *Agriculture, Ecosystems & Environment*, **118**(1–4), 6-28.

Smith, P., Powlson, D., Glendining, M., Smith, J.O. 1997. Potential for carbon sequestration in European soils: preliminary estimates for five scenarios using results from long-term experiments. *Global Change Biology*, **3**(1), 67-79.

Sofo, A., Nuzzo, V., Palese, A.M., Xiloyannis, C., Celano, G., Zukowskyj, P., Dichio, B. 2005. Net CO2 storage in mediterranean olive and peach orchards. *Scientia Horticulturae*, **107**(1), 17-24.

Soil Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. United States Departmentof Agriculture and Natural Resources Conservation Service, Washington, DC.

Syswerda, S.P., Corbin, A.T., Mokma, D.L., Kravchenko, A.N., Robertson, G.P. 2011. *Soil Science Society of America Journal*, **75**(1), 92-101.

Townsend, A.R., Vitousek, P.M., Trumbore, S.E. 1995. Soil Organic Matter Dynamics Along Gradients in Temperature and Land Use on the Island of Hawaii. *Ecology*, **76**(3), 721-733.

Trumbore, S.E. 1997. Potential responses of soil organic carbon to global environmental change. *Proceedings of the National Academy of Sciences*, **94**(16), 8284-8291.

Trumbore, S.E., Zheng, S. 1996. Comparison of fractionation methods for soil organic matter 14C analysis. *Radiocarbon*, **38**(2), 219-229.

Valboa, G., Lagomarsino, A., Brandi, G., Agnelli, A.E., Simoncini, S., Papini, R., Vignozzi, N., Pellegrini, S. 2015. Long-term variations in soil organic matter under different tillage intensities. *Soil and Tillage Research*, **154**, 126-135.

Wäldchen, J., Schulze, E.-D., Schöning, I., Schrumpf, M., Sierra, C. 2013. The influence of changes in forest management over the past 200 years on present soil organic carbon stocks. *Forest Ecology and Management*, **289**, 243-254.

Wiseman, C.L.S., Püttmann, W. 2005. Soil organic carbon and its sorptive preservation in central Germany. *European Journal of Soil Science*, **56**(1), 65-76.

Xu, X., Trumbore, S.E., Zheng, S., Southon, J.R., McDuffee, K.E., Luttgen, M., Liu, J.C. 2007. Modifying a sealed tube zinc reduction method for preparation of AMS graphite targets: Reducing background and attaining high precision. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, **259**(1), 320-329.

Chapter 4- Agronomic use of olive pomace: short and long term effects on soil quality and carbon storage

4.1. Introduction

Olive groves are one of the most important tree crops in Europe and whole Mediterranean area, covering about 7 Mha in Mediterranean area, of which about 5 Mha are included in European countries (Lòpez-Piñeiro et al., 2011) and 1.1 Mha in Italy (Eurostat, 2008). Among European countries, Spain, Italy, Greece and Portugal account for more than 70% of olive oil extraction, that is one of the most traditional and economically important agricultural industry (Owen et al., 2000). As a consequence, these countries produce more than 11 Mt of olive oil waste annually (Lòpez-Piñeiro et al., 2011), a huge quantity of wastes to dispose in a short period of time (Roig et al., 2006). In particular, Italy contribute about for 20% to olive European production (14.0 Mt in the year 2013, as reported by Eurostat) with an olive oil waste production of more than 2000 t y^{-1}. Olive oil extraction by-products are both liquid and solid, and their amount and physical/chemical properties depend on oil extraction method (Roig et al., 2006). In the last few years the olive oil extraction methods are changed from the traditional discontinuous press system to modern centrifugal systems (called three-phase and two-phase systems) in order to improve oil quality and process efficiency, determining changes in by-products with regard to types and composition (Roig et al., 2006). The three-phase system generates two liquid fractions (oil and wastewater) and a solid one (olive pomace), that can be further utilized in the olive-pomace oil extraction process by using organic solvent as hexane (after drying phase). Instead, the two-phase system produce one liquid fraction (oil) and a solid one (two phase olive mill waste, called also *alperujo*, characterized by a higher moisture compared to olive pomace), that cannot be used to produce olive-pomace oil, because of the higher operational costs due to the drying phase, that make the process uneconomical (Alburquerque et al., 2004; Roig et al.,

2006). The high operational costs to process *alperujo*, associated with drastic reduction in consumer demand for olive-pomace oil, due to its low quality, transformed olive pomace into a waste for disposal (Roig et al., 2006) with relevant environmental problems. To face this problem, several methods have been applied for treatment of both olive-mill wastewater and pomace, including ultrafiltration/reverse osmosis (Niaounakis & Halvadakis, 2006), thermal concentration and evaporation (Netti & Wlassics, 1995), incineration and combustion (Vitolo et al., 1999) or combustion and gasification (Caputo et al., 2003) and anaerobic digestion treatments with biogas production (Rozzi & Malpei, 1996). However, these methods do not sufficiently eliminate the pollution problems (Paredes et al., 2002) and their high costs make them not usable in the small-scale of olive mills (Chowdhury et al., 2013). An alternative use of olive-mill by-products may be the field spread, that could be the complete solution to face the problem of waste disposal. In particular, the olive pomace, or the relative pomace compost, could be used in agriculture for organic amendment, an environmentally friendly agronomic practice (Paredes et al., 2005), especially useful for lands under intensive agricultural production, that generally are affected by soil fertility loss, soil erosion, soil compaction and reduction in organic matter content (Chowdhury et al., 2013). The latter condition is very common in Mediterranean soils and highly correlated with soil potential productivity and fertility, due to direct impact of organic matter content on soil physical, chemical and biological properties (Alburquerque et al., 2007).

Italian law (*Legge n. 574/1996*) allows the field spread for agronomic use of both olive pomace and olive-mill wastewater (OMWW), with an amount of 50 or 80 m^3 ha^{-1} y^{-1} (traditional press or continuous oil-extraction systems, respectively). However, the agronomic use of OMWW by directly spread is limited by some constraints, such as oil and grease content (Amaral et al., 2008), high salinity, acidity and the very high organic loads, composed by sugars, polyalcohols, pectins, lipids and significant amount of aromatic compounds, such as tannins and polyphenols

(Chowdhury et al., 2013). In particular, polyphenols are mainly responsible for antimicrobial activity and phytotoxicity of OMWW (Niaounakis and Halvadakis, 2006), as widely documented in literature (Di Serio et al., 2008; Piotrowska et al., 2011; Barbera et al., 2013; Di Bene et al., 2013), furthermore other OMWW characteristics can cause potential negative effects on chemical and biological properties of soils, potential phytotoxic effects on crops, and potential pollution of groundwater (Saadi et al., 2007).

Rodis et al. (2002) estimated that of the total polyphenols present in olives before milling, 2% are found in oil, 53% in OMWW and 43% in pomace. Therefore, we can suppose that also olive pomace could negatively affect soil properties after amendment, but not clear and satisfying results are available in literature about its effects on soils. Generally, olive pomace could be potentially considered a good organic amendment able to improve soil quality in agricultural ecosystems, by increasing aggregates stability, water retention, organic matter content and nutrient availability (Lòpez-Piñeiro et al., 2002), as reported also in degraded or semi-arid soils of Mediterranean area (Lòpez-Piñeiro et al., 2008; Ouzonidou et al., 2010), due to the high content in organic matter, total N and available K and the low content in heavy metal (Pb, Cd, Cr <1 mg kg^{-1}, as reported by Roig et al., 2006, and Hg <0.1 mg kg^{-1}, as reported by Nasini et al., 2013). In addition, Carbonell-Bojollo et al. (2009) also hypothesized that olive pomace amendment can be a valid tool to favour C sequestration in soils, but as regards this aspect, it has to be evaluated if pomace amendment is able to increase organic C in the more stable pools, by analysing C distribution among SOM fractions with different stability. On the other hand, the low pH, the relatively high salinity and the presence of phenolic compounds make olive pomace a potentially harmful substance for the environment, and this aspect deserve to be further investigated. In fact, as concerns implications of this agronomic practice on soil biota, no clear or generalizable conclusion can be deduced by literature, because of the variability in olive mill by-products tested or in biological parameter

assayed (Gomez-Muñoz et al., 2012; Sampedro et al., 2009; Saviozzi et al., 2001). Therefore further studies have to be performed to assess the effects on soil microbial community, that plays a key role in decomposition process and nutrient cycles and strongly affects soil quality.

In this study, we analysed the effect of amendment with olive pomace on soil quality and C stock, both in long (8 years) and short (3 months) term. For this purpose, we assayed a minimum data set of soil chemical and biological parameters (pH, electrical conductivity, organic carbon, mineral and total nitrogen, microbial biomass carbon, fungal-mycelium biomass and potential respiration) and also calculated some microbial indices, such as metabolic quotient (qCO_2), coefficient of endogenous mineralisation (CEM), microbial quotient (both for microbial C and fungal-mycelium biomass), fungal fraction of microbial C and the AI3 index (Puglisi et al., 2006), based on three soil enzyme activities, like useful tools to better evaluate changes in soil quality due to variations in environmental factors. Moreover, we also analysed organic C stock in SOM fractions with different stability, to evaluate if the increase in soil organic C after amendment corresponded to an increase in more stable SOM fractions. Since, in field studies, changes in environmental conditions can greatly affect soil microbial activity and growth, causing variable results, we carried out a laboratory experiment under controlled conditions of soil moisture and temperature. The overall purpose of this research was to provide a more clear and complete analysis of the positive/negative effects of olive pomace amendment in order to better understand if this agronomic practice can be considered environmental sustainable and safe.

4.2 Materials and methods

4.2.1 Study area

Soil tested in the laboratory experiment was collected in an olive grove located in central Italy, near Assisi town (12°56’ E longitude, 43°11’N latitude, and about 400

m a.s.l.). This area is characterized by low precipitations and high temperature during the summer and by low temperature during the winter, and shows an average precipitation value of about 800 mm per year, predominantly concentrated in spring and fall (Nasini et al., 2013). The soil is classified as Typic Haploxere (Soil Survey Staff, 2010), deriving from calcareous marl, and is characterized by alkaline pH (8.1), loam texture (having 41% sand, 33% silt and 25% clay) and a low amount of organic matter, total N, available P and exchangeable K (Nasini et al., 2013).

The olive grove was ten-years-old, non-irrigated and covered by the *Leccino* cultivar (*Olea europea* L.). Since March 2006, in this field, a study was performed to analyse the effect of amendment with olive pomace, from a three-phase oil extraction system, on growth and productivity of olive trees and on oil quality. For this purpose, six plots with five olive trees, that were representative for vegetative and productive characteristics of the entire olive grove, were selected: three plots were yearly amended with 50 t ha^{-1} of pomace (according with Italian low *Legge n. 574/1996*) and three was used as control. Moreover, on treated and control plots, a week after amendment, 100 kg ha^{-1} of urea were spread and, after one month, 1 kg of a complex fertiliser (Nitrophoska blu Spezial NPK 12-12-17) was also applied.

4.2.2 Soil sampling and experimental design

At sampling time (December 2013), 8 years after starting field experiment, soil was collected (from a depth of 0 down to 20 cm) from amended and control plots, in order to constitute two experimental conditions: unamended (UA) and 8 years amended (8y-A). The two pools of sampled soil were sieved at 2 mm mesh and used to fill PVC cylinders (6,5 cm diameter, 18 cm height and closed in the bottom by gauze) to set up the laboratory microcosms, a miniaturized ecosystems useful to investigate, under controlled conditions, the effects of the studied treatments.

On both experimental conditions (UA and 8y-A) five treatments were assayed: 1) control soil; 2) soil + olive pomace (P), 3) soil + olive pomace + urea (P+U), 4) soil + olive-pomace compost (P-C), 5) soil + olive-pomace compost + urea (P-C+U).

Each treatment was triplicate and the number of samples to carry out was planned considering five samplings (at 2, 4, 6, 8 and 12 weeks after treatments application), for a total number of 150 soil cores incubated.

The organic amendments (pomace and olive-pomace compost) were applied on soil surface with a doses of 50 t ha^{-1}, while urea had a doses of 100 kg ha^{-1}.

A schematic representation of the experimental design is shown in the Figure 4.1.

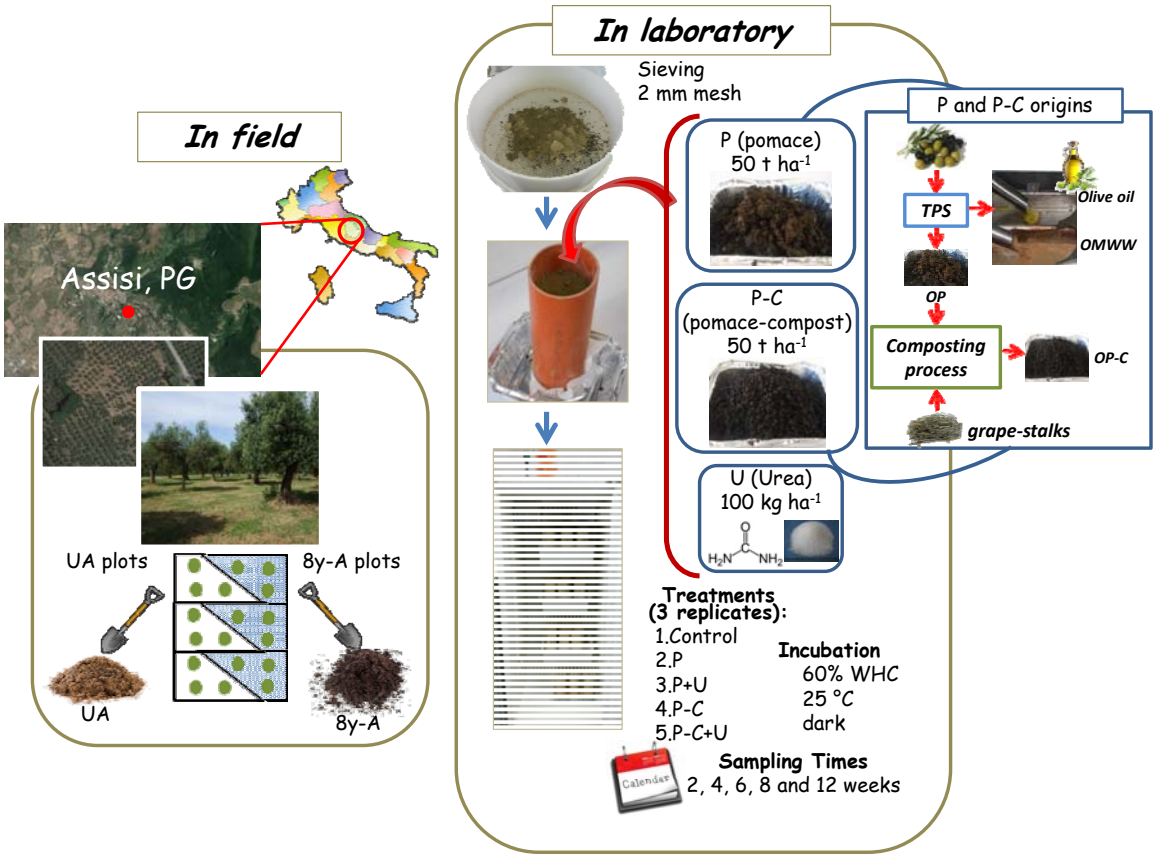


Figure 4.1 Scheme of the experimental design. UA: unamended soil; 8y-A: 8-years amended soil; WHC: water holding capacity; TPS: three-phase system; OMWW: olive mill wastewater; OP: olive pomace; OP-C: olive-pomace compost.

The characteristics of the olive pomace and olive-pomace compost used in the experiment were reported in Table 4.1

Table 4.1 Chemical properties of the olive pomace and olive-pomace compost used in the experiment compared to the set of requirements of Italian law for a generic compost (*D.Lgs 75/2010*, set of requirements for *ammendanti compostati misti*).

Parameter	Pomace	Pomace-compost	Other compost
Moisture (%)	44.1(4.3)	32.4 (1.0)	<50
pH	6.6 (0.22)	8.08 (0.02)	from 6.0 to 8.5
Electrical conductivity (mS cm^{-1})	0.5 (0.13)	-	
Organic Carbon (g kg^{-1})	489.7 (1.6)	425.2 (4.0)	> 200
Water extractable OC (g kg^{-1})	900 (114)	-	
HA + FA (g kg^{-1})	103 (30)	160.49 (10.02)	> 70
Total K and N (g kg^{-1})	-	19.04 ± 0,02	
Total Organic N (g kg^{-1})	-	16.80 (0.04)	> 80% di TKN
Total N (g kg^{-1})	24.2 (1.4)	23.0 (0.9)	
C/N ratio	20.3 (1.1)	18.5 (0.9)	< 25
Seed germination index (%)		94.2 (1.6)	-
Lipids (g kg^{-1})	46 (5.3)	-	
Polyphenols (g kg^{-1})	4.5 (0.4)	-	
Total K (g kg^{-1})	8.9 (1.0)	7.62 (0.2)	-
Total P (g kg-1)	1.2 (0.2)	2.67 (1.9)	-
Total Cu (mg kg^{-1})	50 (11)	41.5 (0.5)	< 150
Total Zn (mg kg^{-1})	24 (6)	63.9 (2.8)	< 500
Total Pb (mg kg^{-1})	<2[a]	-	< 140
Total Cd (mg kg-1)	<0.2a	0.31	< 1.5
Total Ni (mg kg^{-1})	9 (4)	-	< 50
Total Hg (mg kg^{-1})	<0.1[a]	-	< 1.5
Total Cr (mg kg^{-1})	15 (3)	12	
Total Mn (mg kg^{-1})	28 (6)	22	

The soil cores were incubated in the dark, at 25 °C and 60% of water holding capacity (WHC), maintained constant by checking periodically the core weights and adding the water evaporated.

The experiment was planned with the purpose to reproduce in laboratory the experimental conditions of the field study, so doses of pomace and urea were selected according with the quantity applied by Nasini et al. (2013) and the requirements of Italian law (*Legge n. 574/1996*).

Finally, 18 cm-high cylinders were selected because the most intense microbial activity is observed in the superficial horizons (Bertrand et al., 2015).

4.2.3 Soil physical and chemical analyses

Chemical parameters, like pH, EC, organic C and total N, were analysed only at three sampling times (2, 6, 12 weeks after treatments application), while water content and biological parameters were analysed in all sampling times (2, 4, 6, 8 and 12 weeks after treatment application) and WHC was measured only on the two soil pools, before the incubation, in order to incubate soil cores at 60% of WHC.

Water content and WHC were determined by gravimetric method (Allen, 1989). Soil pH and electrical conductivity were measured in 1:2,5 and 1:2 soil:water suspension, respectively, by potentiometric method (ISO 10390, ISO 11265). Organic C and total N were measured by flash combustion method with an CN-soil elemental analyzer (Flash EA2000 Thermo); prior to CN analyses, soil samples were pulverized, up to obtain a fine homogeneous powder, and carbonate removal was performed by fumigation with HCl method (Harris et al., 2001); to determine the percentage of organic C and total N, a calibration rate was calculated using BBOT standard (6.51 % N, 72.53 % C, 6.09 % H, 7.44 % S) using a linear regression as calibration method.

Mineral-N was calculated as the sum of NO_3^--N (nitric N) and NH_4^+-N (ammoniacal N), that were measured by potentiometric method after soil extraction by K_2SO_4 0.5 M (Castaldi & Agarosa, 2002) utilising ion-selective electrodes for ammonia (NH_4^+-

N) (Model Orion 9512) and nitrate N (NO_3^--N) (Model Orion 9707) connected to a pH/ISE meter (ORION, Model 290A).

4.2.4 Soil biochemical and biological analyses

The methods to assay the soil microbial community includes measures of microbial biomass carbon, respiration and enzymatic activities (Kieft et al., 1998).

Basal respiration was measured as CO_2 evolution from fresh soil samples in time. Soil samples (4 g fresh soil) were moistened at 55% WHC with deionised water and placed into 30 ml vials closed by butyl caps and aluminium seals, then the head space gas was ventilated with standard concentration air (20% O_2, 80% N_2) for 2 minutes. Finally the samples were incubated for 1 h at 25°C, at dark and at the end of this incubation period, the headspace gas was sampled by a syringe and CO_2 concentration was measured by PP-system equipped with IRGA detector (EGM-4 instrument, static measurement). The ppm CO_2 concentration values recorded by instrument were converted opportunely firstly in ml CO_2 h^{-1} g^{-1} dry soil, knowing vials volume, and then in mg CO_2 h^{-1} g^{-1} dry soil by ideal gas law:

$$pV=nRT$$

The microbial biomass carbon (MBC) was determined by SIR method (Substrate Induced Respiration), that was proposed by Anderson and Domsch (1978) and based on the respiratory response of soil microbial biomass to the fresh addition of a readily available substrate, such as glucose, in controlled conditions of moisture and temperature. As reported by method, we tested different D-glucose concentrations (i.e. 50, 100, 200 μmol of glucose g^{-1} dry weight of soil) and different times of incubation (1, 2, and 3 h after glucose addition) to establish the maximal initial respiration rate, that in our soils was obtained with a D-glucose concentration of 50 μmol g^{-1} and after 2 hours of incubation. Thus, potential respiration was measured similarly to basal respiration, adding to samples D-glucose and incubating at 55% WHC, 22°C and in the dark for 1 h (measures were carried out in the second hour

after glucose addition). The CO_2 produced during the incubation period was converted into soil MBC by the equation reported by Anderson and Domsch (1978) finding a relation between the amount of MBC by SIR and that obtained by fumigation and incubation method (Jenkinson et al., 1979):

$$MBC\ (mg\ Cmic\ g^{-1}\ ds) = 40.04y + 0.37$$

where y is the maximal initial respiration rate (ml CO_2 g^{-1} d.w. h^{-1})

The fungal mycelium was determined at optical microscope by the membrane filter technique (Sundman & Sivelä, 1978) and the intersection method (Olson, 1950), staining the membrane filter with aniline blue in 80% lactic acid. The mass of total mycelia was calculated on the basis of the average values of cross section (9.3 μm^2), density (1.1 g ml^{-1}) and dry mass of the hyphae (15% of the wet mass) according to Berg & Wessén (1984). Fresh soil (1 g) was homogenized with 100 ml of deionised water (6000 rpm for 2 minutes), then 0.5 ml of this suspension were filtered on cellulose nitrate membrane filter (porosity 45μm) and stained by aniline blue.

4.2.5 AI3 index and microbial indices

The AI3 index was proposed and validated by Puglisi et al. (2006) as an useful tool to evaluate changes in soil quality due to environmental factors, like salt, water irrigation, heavy metal contamination, erosion and organic fertilization.
The authors reported that, in general, altered soil had higher index scores than unaltered soils. The index AI3 was calculated as follow:

$$AI3 = 7.87\ \beta\text{-glucosidase} - 8.22\ \text{phosphatase} - 0.49\ \text{urease}$$

The β-glucosidases activity was measured applying the method proposed by Eivazi & Tabatabai (1977); urease activity was measured by Kandeler & Gerber (1988) method and phosphatase activity was determined as proposed by Eivazi and

Tabatabai (1988). Data of enzyme activities were provided by Fioretto *et al.* (unpublished data).

The metabolic quotient (qCO_2) reflects the maintenance energy requirement of soil microbes (Anderson, 2003), and can be a relative measure of how efficiently the soil microbial biomass is utilizing C resources to increase its biomass, as well as the degree of substrate limitation for microbial growth (Wardle & Ghani, 1995; Dilly & Munch, 1998). Anderson (2003) indicated above 2 mg C-CO_2 h^{-1} g^{-1}MBC as a critical threshold for the "baseline performance" of microbial community. It is calculated as follow:

$$qCO_2 = \frac{\text{mg C} - CO_2\ h^{-1}}{\text{g MBC}}$$

The coefficient of endogenous mineralization (CEM) represents fraction of organic carbon mineralized to CO_2 in time, and provides information about organic matter mineralization and potential accumulation or losses of organic carbon in soils. Generally has been observed that CEM increases in soil after a stress condition (D'Ascoli et al., 2005; Gijsman et al.,1997; Rutigliano et al., 2004). It is calculated as follow:

$$\text{CEM} = \frac{\text{mg C} - CO_2\ h^{-1}}{\text{g } C_{org}}$$

The microbial quotient (q_{mic}) represents microbial fraction of soil organic carbon (Anderson & Domsch, 1989), being the part of organic C with the fastest turnover. It can be also calculate by using fungal mycelium biomass data. It could be used as stability indicator for a quick recognition of environmental changes (Anderson, 2003), in fact this quotient has been proposed as a sensitive indicator of quantitative changes in SOM due to change in management and climate (Anderson & Domsch, 1989). The ratio is expressed by:

$$q = \frac{C_a}{OC} = \frac{mg\ C_a\ g^{-1} d.\ s.}{g\ OC\ g^{-1} d.\ s}$$

where C_a is the organic C of microbial biomass or fungal biomass.

The fungal fraction of the microbial carbon (C_{fung} $\%C_{mic}$), represented the relative abundance of fungal component in soil microbial community, and was obtained converting the weight of total mycelia into weight of fungal carbon, on the basis of mean values reported for fungal C/N ratio (Killham, 1994) and fungal N content (Swift et al. 1979), and was expressed as percentage of total microbial carbon.

4.2.6 SOM fractionation and pMC (^{14}C) in stabile fractions

Soil organic matter fractionation was performed by a physical-chemical fractionation method (Castanha et al., 2008; Marzaioli et al., 2010; Trumbore & Zheng, 1996), as reported in details in Chapter 3. It was performed on six replicates for each experimental condition (UA and 8y-A), in order to identify how the organic C inputs derived from the organic amendment had been incorporated in the different SOM fractions. The soil organic matter fractions HF and REC and also the bulk soil were analyses for radiocarbon content as percent of modern carbon (pMC) by AMS previous a graphite conversion of each samples, following the same procedure reported in Chapter 3.

4.2.7 Statistical analysis

For each chemical or biological parameter, mean and standard deviation were calculated on the three replicates for each treatment in both experimental conditions and at each sampling time. Significant differences among treatment were tested by one way ANOVA, followed by the Student-Newman-Keuls test or Dunnet test ($P<0.05$; SigmaPlot 12.0). Linear correlations among the parameters were also calculated by Pearson coefficient ($P<0.05$; SigmaPlot 12.0).

4.3 Results and Discussion

4.3.1. Short and long term effects of olive pomace amendment on soil chemical properties

In Tables 4.2 and 4.3 are reported soil chemical properties in the two experimental conditions (UA and 8y-A) and for each treatment, during the period of incubation at three time of sampling: 2, 6 and 12 weeks after treatment application.

Soil pH did not show remarkable effects due to treatments in short term. In fact no significant difference was observed between each treatment and control soil, in both experimental conditions. However, 8 years of yearly amendment with olive pomace caused a low but significant decrease in soil pH, as shown by the slightly alkaline pH in 8y-A compared to the moderately alkaline pH measured in UA soil (USDA, 1993). Electric conductivity (EC) showed some changes (Table 4.2) but not clearly related to treatments in the short term. On the contrary, significant higher values (+76% on average) were observed in 8y-A soil in comparison with the UA soil ($P<0.05$). The mineral N content (Table 4.2), evaluated as sum of NO_3^--N and NH_4^+-N, showed some variations among treatments during incubation in both experimental conditions, and in particular, the mineral N content in soil samples treated with pomace was always significantly lower compared to control ($P<0.05$). Moreover, mineral N content in 8y-A soil was significantly higher (three times on average) than in UA soil. In both experimental conditions (UA and 8y-A soils), organic C and total N contents were not affected by treatments in the short term (Table 4.3), instead on the long term, after 8 years of pomace amendment, a significant increase in organic C and total N ($P<0.05$) were found compared to unamended condition. The C/N ratio did not changed in short term too, but it was surprisingly reduced in 8y-A soil compared to UA soil, indicating that the increase in total N due to amendment exceeded the increase in organic C.

Table 4.2 Mean values (± standard deviations) of soil pH, electrical conductivity (EC) and mineral nitrogen (measured as sum of NH_4^+-N and NO_3^--N) at 2, 4 and 6 weeks after treatment application. Significant differences ($P<0.05$) among treatments, in the same time of sampling, are indicated by different letters in apex; significant differences between two experimental conditions (UA and 8y-A), in the same time of sampling, are indicated by an asterisk.

	pH			**EC (µS cm^{-1})**			**Mineral N (µgN g^{-1})**		
Treatments	2 weeks	6 weeks	12 weeks	2 weeks	6 weeks	12 weeks	2 weeks	6 weeks	12 weeks
UA control	8.05 ±0.12	8.12 ±0.05	8.25 ±0.01	476.67^{a} ±3.01	482.83 ±5.58	548.00^{a} ±71.23	21.19^{a} ±1.20	22.95^{a} ±1.31	32.79^{a} ±2.92
UA+P	8.09 ±0.05	7.99 ±0.06	8.29 ±0.06	483.00^{ab} ±13.81	477.50 ±21.55	430.50^{b} ±9.10	13.54^{b} ±0.82	12.29^{b} ±0.12	15.42^{b} ±1.80
UA+P+U	8.06 ±0.04	8.06 ±0.02	8.21 ±0.11	581.33^{b} ±47.02	491.00 ±5.29	566.17^{ab} ±91.82	17.59^{c} ±2.32	24.83^{a} ±7.49	27.61^{ac} ±2.99
UA+P-C	7.99 ±0.03	7.92 ±0.19	8.47 ±0.28	568.33^{a}b ±75.96	487.33 ±2.89	483.25^{a} ±30.76	16.07^{c} ±1.16	24.23^{a} ±1.59	23.44^{c} ±3.65
UA+P-C+U	8.00 ±0.02	8.06 ±0.06	8.21 ±0.05	514.17^{b} ±12.77	498.33 ±18.51	434.00^{ab} ±5.22	21.89^{a} ±0.40	32.28^{a} ±4.26	27.68^{ac} ±2.60
8y-A control	7.69* ±0.03	7.69* ±0.07	7.72* ±0.07	761.33^{A}* ±10.79	864.67^{A}* ±67.28	901.67* ±108.29	67.74^{AB}* ±7.41	71.03^{A}* ±5.66	94.86^{A}* ±2.91
8y-A +P	7.67 ±0.04	7.62 ±0.09	7.84 ±0.03	768.83^{A} ±16.54	722.00^{B} ±24.11	833.17 ±68.23	52.68^{B} ±6.89	57.75^{B} ±7.35	71.26^{B} ±4.78
8y-A+P+U	7.65 ±0.10	7.73 ±0.08	7.76 ±0.08	788.17^{A} ±56.20	796.83^{B} ±22.31	955.50 ±109.30	61.51^{B} ±9.94	67.60^{A} ±4.02	78.43^{B} ±4.09
8y-A+P-C	7.63 ±0.11	7.67 ±0.04	7.81 ±0.02	814.33^{A} ±8.69	766.17^{B} ±14.25	766.00 ±51.97	71.70^{AB} ±7.97	70.63^{A} ±4.87	87.77^{A} ±8.16
8y-A+P-C+U	7.53 ±0.07	7.72 ±0.08	7.71 ±0.06	873.00^{B} ±31.78	740.17^{B} ±13.53	868.00 ±33.61	82.01^{A} ±5.95	74.21^{A} ±2.37	92.13^{A} ±5.50

Table 4.3 Mean values (± standard deviation) of organic C (OC) and total N (TN) content and C/N ratio measured 2, 4 and 6 weeks after treatments application. Asterisk indicate significant differences (P<0.05) between the two experimental conditions.

	OC (%)			TN (%)			C/N		
Treatments	2 weeks	6 weeks	12 weeks	2 weeks	6 weeks	12 weeks	2 weeks	6 weeks	12 weeks
UA control	1.11 ±0.13	1.36 ±0.02	1.20 ±0.14	0.10 ±0.02	0.11 ±0.01	0.12 ±0.07	11.58 ±3.95	15.72 ±4.22	11.73 ±5.26
UA+P	1.36 ±0.04	1.68 ±0.32	1.46 ±0.09	0.12 ±0.00	0.12 ±0.01	0.08 ±0.01	11.45 ±0.46	14.45 ±3.36	17.87 ±1.25
UA+P+U	1.30 ±0.26	2.13 ±0.30	1.46 ±0.12	0.12 ±0.00	0.13 ±0.01	0.09 ±0.01	11.06 ±2.05	15.99 ±2.84	17.04 ±1.28
UA+P-C	1.45 ±0.18	1.86 ±0.17	1.42 ±0.06	0.13 ±0.00	0.18 ±0.07	0.09 ±0.00	11.01 ±1.06	11.41 ±3.57	15.62 ±0.41
UA+P-C+U	1.41 ±0.16	1.83 ±0.03	1.57 ±0.05	0.12 ±0.00	0.14 ±0.00	0.10 ±0.00	11.37 ±1.30	11.55 ±3.05	16.52 ±0.50
8y-A control	2.35* ±0.09	2.35* ±0.15	2.48* ±0.21	0.24 * ±0.01	0.24 * ±0.00	0.25 * ±0.02	9.70 * ±0.31	9.68 * ±0.68	9.82 * ±0.21
8y-A +P	2.75 ±0.19	2.80 ±0.12	2.62 ±0.17	0.26 ±0.00	0.26 ±0.01	0.29 ±0.02	10.49 ±0.56	10.78 ±0.49	9.16 ±0.16
8y-A+P+U	2.59 ±0.17	2.79 ±0.07	2.75 ±0.25	0.26 ±0.02	0.25 ±0.01	0.31 ±0.00	10.02 ±0.19	11.10 ±0.28	9.41 ±0.04
8y-A+P-C	2.88 ±0.23	2.43 ±0.17	2.70 ±0.10	0.28 ±0.01	0.25 ±0.01	0.30 ±0.00	10.12 ±0.81	9.63 ±0.36	9.09 ±0.35
8y-A+P-C+U	2.99 ±0.11	2.38 ±0.40	2.83 ±0.09	0.29 ±0.00	0.24 ±0.00	0.28 ±0.01	10.42 ±0.33	10.03 ±1.62	10.24 ±0.53

Our results about soil pH were in agree with Brunetti et al. (2005) that, after three months of application, in crop land, of “crude olive pomace” (from three phase system) and “exhausted pomace” (obtained after further extraction of the crude one) no variation in soil pH found, with the pomace-amended soils and control that showed the same moderately alkaline pH. In this study, after 8 years with pomace amendment a lower but significant decrease in soil pH was found, and this decrease

in soil pH was probably due to phenolic and carboxylic group resulting from organic matter decomposition of the olive pomace and olive pomace compost, which act as weak acids (García-Ruiz et al., 2012) or more in general to the pH of the pomace amendment. In fact in olive pomace a moderately low pH is typically observed (Table 4.1). However, contrasting results are reported in literature about pH variations after more or less long period of organic amendment of soil. For example, after three years of amendment, no change in pH was observed by Nasini et al. (2013) in amended plots, similarly after five years of amendment with pomace, Lòpez-Piñiero et al. (2008) reported only a weak and not significant decrease in soil pH (between 0.12 to 0.17 units), and also Camposeo & Vivaldi (2011) observed that de-oiled pomace, used as organic material for soil mulching in super high-density olive groves, did not affect soil properties after two years. Therefore, variations in soil pH could be function of the length of amendment. In fact, García-Ruiz et al. (2012), in a study on the evaluation of the cumulative effect of repeated pomace-compost applications in different olive groves of Spain, observed that the soil pH was reduced from 0.23 to 1.47 units during a period of amendment ranging from 4 to 16 years, while no change were measured for olive groves amendment shorter than 4 years. Instead, literature finding about the effects of olive-mill waste water spread, reported that generally the soil pH remains nearly constant after the application despite the high acidity (Barbera et al., 2013), with only small and temporary variations (Piotrowska et al., 2011; Chartzoulakis et al., 2010). Therefore it is possible to affirm that generally weak change of pH are measured, and that the effects are more strongly related to the soil pedological characteristics, such as clay and carbonates contents, and initial pH, that determine the soil buffer capacity (Tisdale et al., 1999). In fact the acid pH of the olive mill waste will result in a long-term loss of carbonate from the top soil (Mahmoud et al., 2010) that at the same time buffer the soil against acidification, for this reason soil rich in lime are preferred for olive mill wastewater addition (Mekki et al., 2009).

Our EC data, in the short period, accorded with those of Brunetti et al. (2005), that after three months from olive pomace application in field did not observed variations in EC, while the results of long term effect were in agree with Lòpez-Piñiero et al. (2008, 2011) that observed an increase in EC after five years of olive pomace amendment (two-phase and de-oiled two-phase olive mill waste), effects documented in literature also as consequence of soil application of olive-mill waste water (Di Serio et al., 2008). However, in literature not every time this effect is reported, such as Camposeo & Vivaldi (2011), that did not record difference in EC between amended and control soil after the application of de-oiled pomace as mulching. Generally, the increase in EC is considered a normal but not positive effect derived from the use of organic amendments in agricultural soils (Bonanomi et al., 2014), due to release of soluble organic and inorganic species during the humification of olive pomace and to the increase of nutrient availability in amended soils, that can negatively affect root water absorption and plant growth (Bonanomi et al., 2014). In fact, in some cases, EC was correlated positively with available P, K and NO_3^- (Lòpez-Piñiero et al., 2011). The degree of change depends on the rate of application, and the effects could be reduced by leaching action of the rain that reduce soil EC transferring salts to the groundwater (Sierra et al., 2007). However, in both UA and 8y-A soils, EC values were higher than those measured in other Italian soils from olive groves (Marzaioli et al., 2010) or from other cultivations amended with compost (Bonanomi et al., 2014).

The mineral N content in soil samples treated with pomace was always significantly lower compared to control, but mineral N content in 8y-A soil was on average three times higher than in UA soil. Similar results were found by Scotti et al. (2015), showing a doubling nitric N content in cropland amended by compost, compared to control, at two years after addition. It has to be emphasized that mineral N content found in UA soil was on average comparable with mineral N content found by Marzaioli et al. (2010) in olive groves of Southern Italy.

During the three months of incubation the OC and TN content were not affected by soil treatments and this is in agree with those suggested by some authors, about the difficulty to record immediately after the organic amendment an improve in carbon and nitrogen stocks (Scotti et al., 2015), because of the solid materials added as amendment after few months appeared still undecomposed and unaltered, and, for this reason, are removed by sieving the soil at 2 mm mesh when the soil samples are processed in laboratory before the analyses. However, Brunetti et al. (2005) observed, only after three months, an increase in organic C content and C/N ratio, probably because in their field study the olive pomace was incorporated for 20 cm in soil. The improvement of C and N reservoirs in soil, after several year of amendment, is widely documented in literature, in fact it was shown by Lòpez-Piñiero et al. (2008, 2011) and Lozano-García et al. (2013), that two-phase olive pomace application significantly increased the soil organic C content, principally in top layer. In particular, the range of variation in SOC, resulted from yearly amendment in the study of Lozano-García et al. (2013), was comparable to that found in our study. In fact, in Ap horizon, after three years of amendment, they measured an increase in the SOC content from the value of ~8.9 g kg^{-1} (considered a typical value of Mediterranean agricultural soils) to the value of 18.5 g kg^{-1}, whereas in our case the organic C content varied from ~11 g kg^{-1} to 23 g kg^{-1}. In literature was reported that olive- pomace compost also determined a similar result, in fact Fernández-Hernández et al. (2014) and García-Ruiz et al. (2012) observed a significant increase in soil organic matter in olive grove amended with different kinds of olive pomace compost, with a change in organic matter content, in 4, 9 and 16 years amended soils, from 2.1 to 8.5 times greater than those in untreated control soil (García-Ruiz et al., 2012). However, in the short period of observation, no appreciable variation in organic carbon content can be generally recorded, as reported by Camposeo & Vivaldi (2011) after two years of de-oiled pomace mulching application and by Nasini et al. (2013) after 4 years of addition of pomace from a three-phase oil extraction system, even if

in some study (Clemente et al., 2007b; Montemurro et al., 2004) a significant increase in soil OC was observed in cropland after only one or two year of olive pomace compost or olive pomace amendment. The increase in organic C content is one of the purpose of the organic amendments, as well as compost and olive pomace, especially in semiarid condition, where agricultural soils are often poor in organic matter and subject to intensive degradation, such as Mediterranean olive groves (García-Ruiz et al., 2012; Lòpez-Piñiero et al., 2008; Antolìn et al., 2005). These soils, as consequence of the environmental conditions, are generally subjected to a high mineralization rate and exposed to intensive erosion processes (Fleskens & Stroosnijder, 2007), for this reason the improvement of soil properties, by amendment with olive mill residues, is more important than mineral fertilizer application (Montemurro et al., 2004). Moreover, Carbonell-Bojollo et al. (2009) have considered this agronomic practice as valid tool to favour C sequestration in soils, in accordance with the Kyoto Protocol. The presence of chemically recalcitrant compounds in pomace amended soils, that in part are constituted by the olive by products and in part are formed during composting process (-COOH, phenolic OH contents, and aromatic compounds; Plaza et al., 2007) might contribute to the reduction of the decomposition rate at field condition and consequently can favour C accumulation in soil (García-Ruiz et al., 2012).

Similarly to organic C content, the laboratory treatment did not affect the total N content in both experimental condition, while in the comparison between the UA and 8y-A soil clearly appeared that the field amendment produced a significant increase ($P<0.05$) in soil TN content. In literature, in the short period, no significant variation was generally observed after olive pomace amendment (Brunetti et al., 2005) and, in some cases, also in medium period (three years), after de-oiled pomace application (Camposeo & Vivaldi, 2011). Other findings reported that in olive groves total N significantly increased in plots amended with two-phase olive pomace and with different types of olive-pomace compost, during a period from 3 to 5-6 consecutive

years of amendment (Lozano-García et al., 2013; Lòpez-Piñiero et al., 2008; Fernández-Hernández et al., 2014). Therefore, to record a significant increase in TN, several years of organic amendment are necessary, as reported by García-Ruiz et al. (2012), that in agree with their measure of organic C, also the TN increased when olive pomace compost was applied for more than 9 years.

Literature results highlighted that the prolonged organic amendment with solid olive mill waste, like pomace and its compost, affected soil chemical-physical parameters from the fourth/fifth year (Camposeo & Vivaldi, 2011; Brunetti et al., 2005; López-Piñeiro et al, 2008; Montemurro et al., 2004, García-Ruiz et al., 2012 Fernández-Hernández et al., 2014). In particular, these effects were positive for organic C and total N content, water holding capacity, porosity, bulk density, K availability (Lòpez-Piñiero et al., 2011; Camposeo &Vivaldi, 2011), and for mineral N concentration, but the two main aspects to control were soil pH and EC that, in relation to soil specific conditions and properties, may produce negative effects on soils and plants.

4.3.2. Short and long term effects of olive pomace amendment on soil biochemical/biological properties, microbial indices and AI3 index

The temporal dynamic, in the short term, of each parameter in response to treatments is reported in Figure 4.2, 4.3, 4.5, and the temporal dynamic, in the long term, of the controls, for a comparison between the two experimental conditions (UA *vs* 8y-A), is reported in Figure 4.4.

Microbial biomass C (MBC; Figure 4.2 a) was significantly increased by P and P+U treatments; this effect appeared yet two weeks after the beginning of incubation and lasted until the end of experiment. The same effect was observed in 8y-A soil for P and P+U treatments (Figure 4.2 f), but in this case, despite of the later appearance (since the 6th week), the increase in MBC was more marked than in the UA soil.

Soil fungal-mycelium (Figure 4.3 c, h) did not appeared influenced anyway by treatments; in fact, looking the entire 3 moths dynamic, no differences in fungal mycelium biomass was observed among treatments compared to control, in both

experimental conditions UA and 8y-A, with the only exception of P-C treatment in 8y-A soil, that negatively affected fungal mycelium compared to control.

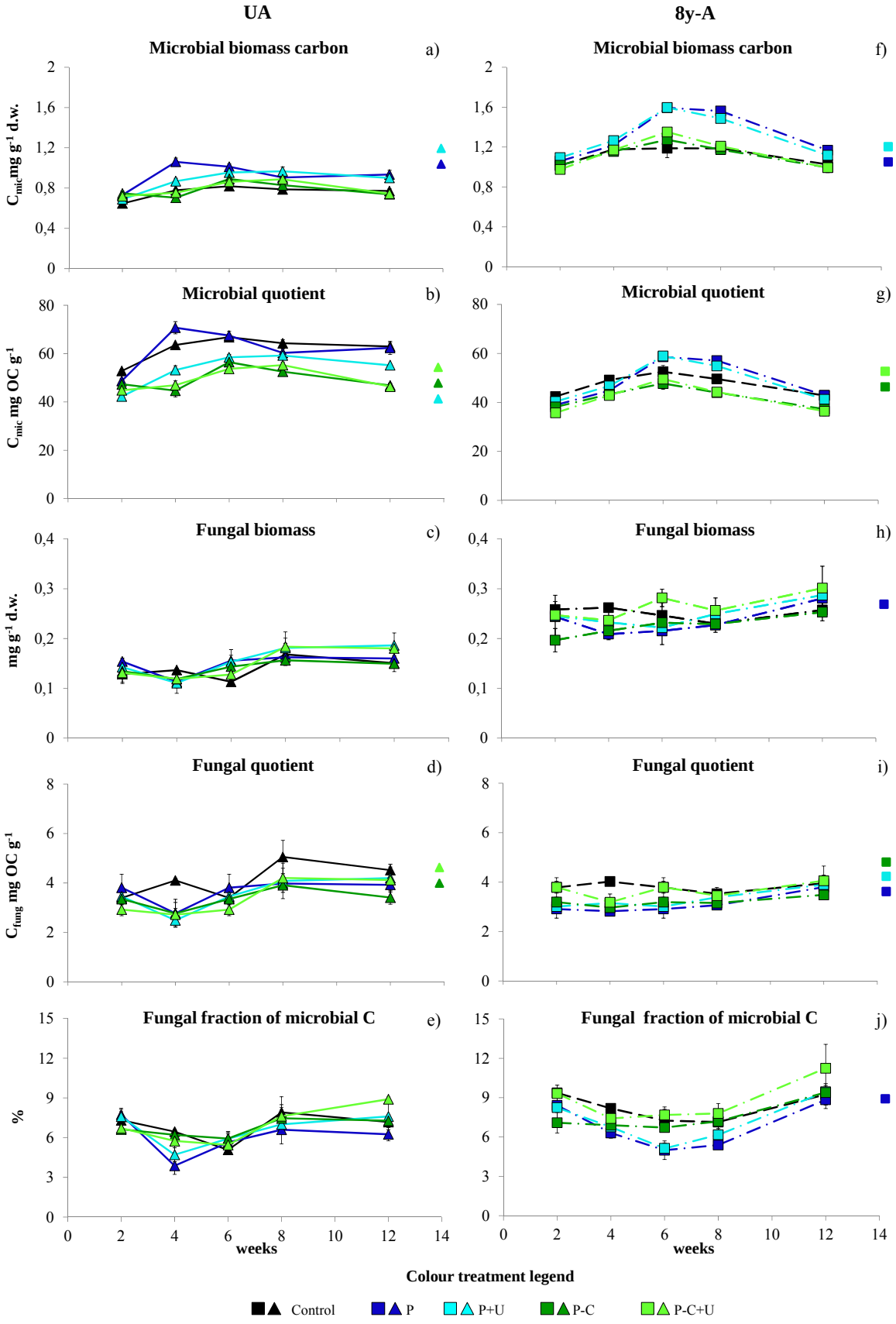


Figure 4.2 Mean (± standard errors) of biological parameters (microbial growth) for unamended soil (UA) in left side (a, b, c, d, e) and 8-years amended soil (8y-A) in right side (f, g, h, i, j). The

coloured simbols on the right sides of the graphs indicate significant differences ($P<0.05$) between the control and each treatment indicated by the colour.

The fungal quotient (qfung) was also reduced after the treatments with pomace-compost (P-C and P-C+U) in UA soil, and after the treatment with P, P+U and P-C in 8y-A soil (Figure 4.2 d, i).

The fungal fraction of microbial C (Figure 4.2 e, j) was not generally affected by treatments, with the except of P treatment that significantly depleted this index in 8y-A soil. In fact, in this study, the increase in MBC was not followed by an increase in fungal biomass, and, as consequence, the fungal-biomass on microbial biomass ratio decreased.

Soil respiration (Figure 4.3 a, b) was stimulated, in the short term, by some treatments in both UA and 8y-A experimental conditions. In particular, significant increases of respiration, compared to control, were observed after P and P+U treatments, in both soils, during the entire observation period; moreover, a significant increase was also observed after P-C treatment, but in 8y-A soil only.

According with respiration data, in both UA and 8y-A soils, metabolic quotient (qCO_2; Figure 4.3 b, e) showed the highest values in the first sapling time, then it decreased, but never below 3 $C\text{-}CO_2$ g^{-1} MBC h^{-1}. However, no difference was observed among treatments and control in both experimental conditions. These results suggested that, at the starting time of incubation (i.e. after two weeks of incubation), in all the treatments (included control) soil microbial community quickly responded to the changes in the environmental conditions (soil temperature and water content) by increasing its activity, while in the following sampling times the quotient decreased as a consequence of the decreased basal respiration rate (Figure 4.2 a, d), probably due to the depletion in easily degradable organic substrate available in soil.

As concerns the coefficient of endogenous mineralization (CEM; Figure 4.3 c, f), in UA soil was only observed a significant reduction in P-C+U treatment compared to control, while in 8y-A soil no difference was observed between control and treatments.

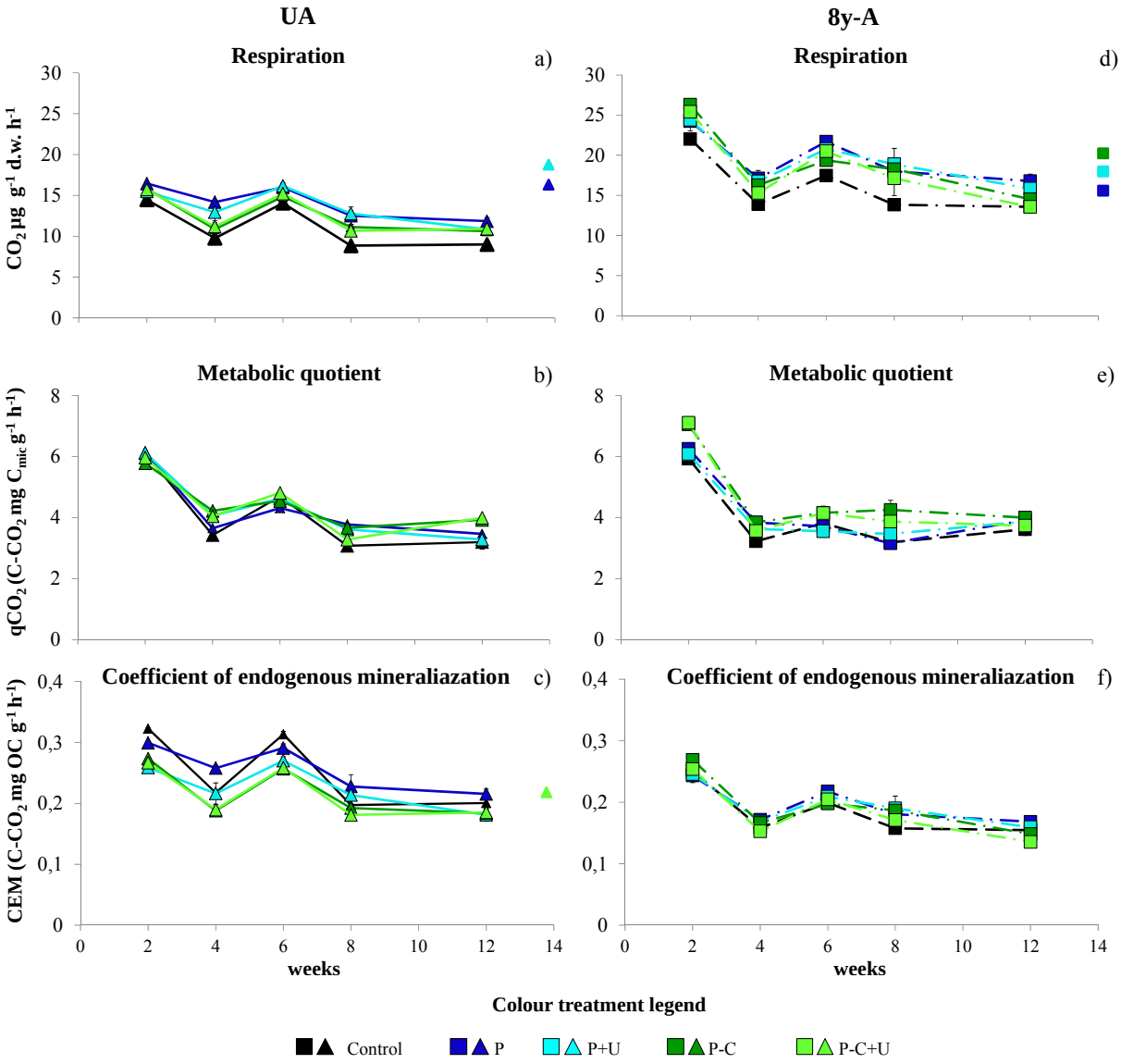


Figure 4.3 Mean (± standard errors) for the respiration activity and relavive microbial indices in unamended soil (UA), on the left side (a, b, c), and in field amended soil (8y-A), on the right side (d, e, f). The coloured symbols on the right sides of the graphs indicate significant differences ($P<0.05$) between the control and each treatment indicated by the colour.

In Figure 4.4, the temporal dynamic in the long term is showed, as comparison between controls of the two experimental conditions (UA *vs* 8y-A).

The long term amendment caused, in 8y-A compared to UA soil, a significant increase in microbial biomass C, fungal biomass, fungal fraction of microbial C and basal respiration (Figure 4.4 a, d, f and g, respectively); on the contrary, significant lower values were measured for microbial quotient and coefficient of endogenous mineralization (Figure 4.4 b and i, respectively), but no difference was observed between the two experimental conditions for fungal quotient and metabolic quotient (Figure 4.4 e and h).

These results indicated that, although pomace amendment stimulated microbial growth and activity, in long term the development of fungal mycelium was more marked than that of MBC, because the fungal fraction of microbial ratio was higher in 8y-A than UA soil. Moreover, the yearly supply of fresh organic matter as pomace did not determine a growth of microbial community proportional to the increase in organic C, in fact the microbial quotient was lower in 8y-A compared to UA soil. Finally, the increase in soil respiration after pomace amendment did not correspond to net loss of organic carbon from soil, because the CEM was lower in 8y-A than in UA soil.

During the experiment three enzyme activities were also measured: β-glucosidase, urease and phosphatase (Fioretto et al., unpublished data). These three enzyme activities were utilized to calculate the AI3 index (Figure 4.5), as proposed by Puglisi et al. (2006), where lower are values higher is soil quality.

In UA soil, no differences in AI3 index among treatments and control were observed (Figure 4.5 a), while in 8y-A soil a significant decrease of AI3 index was observed in P+U, P-C and P-C+U treatments (Figure 4.5 b).

Moreover, a significantly lower value of AI3index was found in 8y-A compared to UA soil (Figure 4.5 c), showing a general higher quality of 8y-A compared to UA soil.

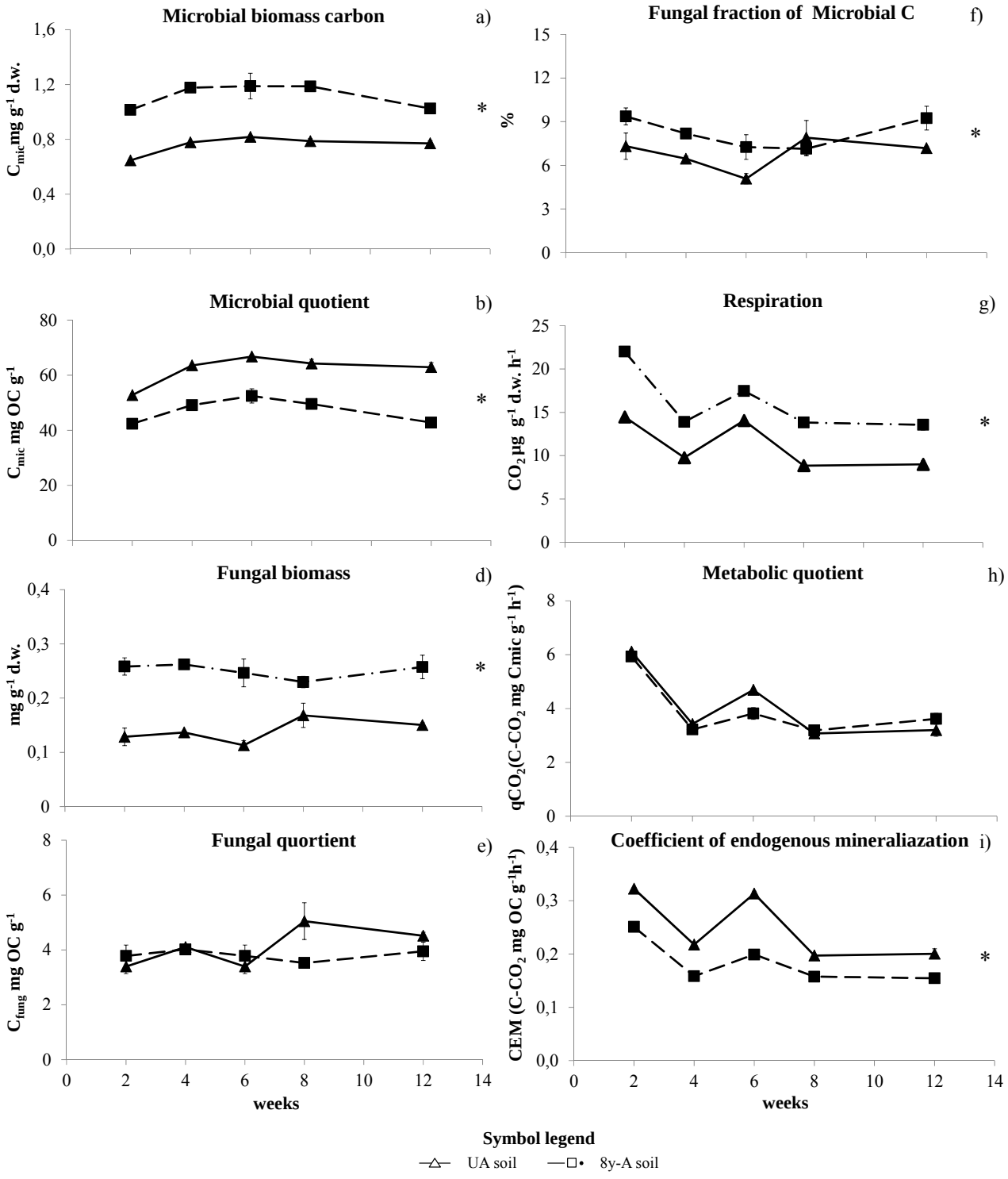


Figure 4.4 Comparison between the controls of the two experimental conditions (mean values± standard errors) for all biological parameters assayed. The asterisks on the right sides of the graphs indicate significant differences (P<0.05) between UA and 8y-A soil.

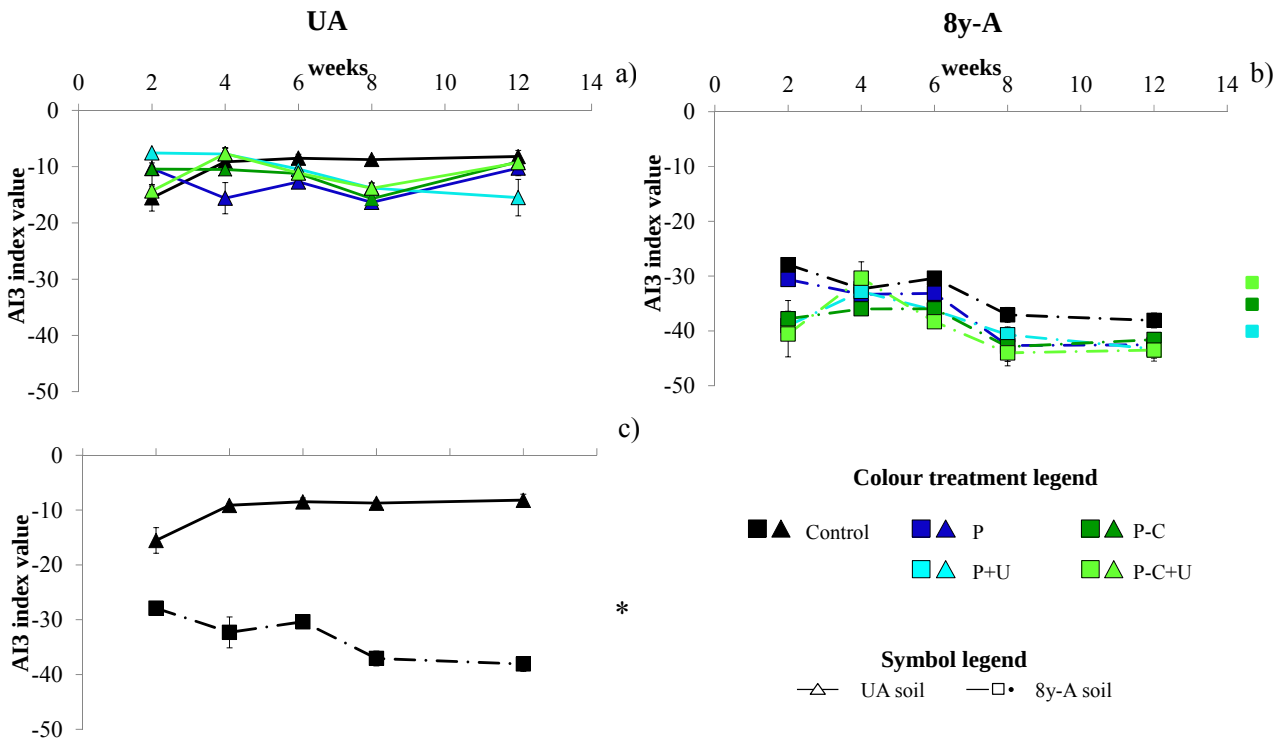


Figure 4.5 Mean values (±standard errors) of AI3 index in unamended soil (UA), on the left side, and in 8-years amended soil (8y-A), on the right side, and the comparison between the controls of the two experimental conditions (UA *vs* 8y-A). The colored symbols on the right sides of the graphs indicate significant differences between control and treatment ($P<0.05$), the asterisks on the right sides of the c) graph indicate significant difference between the two experimental conditions ($P<0.05$).

Regarding Pearson linear correlations among parameters, in UA soil, MBC was positively correlated with OC and C/N ratio ($r^2= 0.537$, $P< 0.001$; $r^2= 0.377$, $P<0.05$, $n= 45$, respectively) on the contrary fungal fraction of microbial C was negatively correlated with OC ($r^2= -0.304$, $P< 0.05$, $n= 45$); these results indicated that in this soil the increase in OC and in C/N ratio stimulated the growth of MBC, but not the growth of fungal mycelium, so to an increase of OC an increase of MBC is related and consequently the Cfun/Cmic ratio decreased. Soil respiration was correlated negatively with mineral N and C/N ratio ($r^2= -0.466$, $P<0.01$; $r^2= -0.369$, $P< 0.01$, $n=45$, respectively) and positively with total N ($r^2= 0.390$, $P< 0.01$, $n= 45$). These correlations could be explained if we considered that microbial activity also depended on nitrogen availability in soil, therefore if total N increased (and thus C/N ratio

decreased) microbial activity was stimulated. However, following this reasoning, an increase in mineral N also stimulated respiration, but the two parameter were negatively correlated; to explain this effect we have to consider that during the study was recorded a decrease in nitric N (and thus in mineral nitrogen) after pomace amendment whereas soil respiration increased. Surprisingly, soil respiration was not correlated with microbial biomass C, because during the incubation the variations of these two parameters followed a different temporal trend. In both UA and 8y-A soils respiration was positively correlated with qCO_2 (r^2= 0.788, P<0.0001; r^2= 0.830, P<0.0001, n= 75, respectively) and with CEM (r^2= 0.852, P<0.0001; r^2= 0.975, P<0.0001, n= 75, respectively) and consequently qCO_2 was positively correlated with CEM (r^2= 0.745, P<0.0001; r^2= 0.838, P<0.0001, n, 75); these results highlighted the strong dependence of qCO_2 and CEM from respiration rather than from MBC or OC.
Pearson linear correlations were also calculated by using exclusively all data of controls for both soils (Table 4.4). Results showed that EC, mineral N, OC and TN were positively correlated each other, but negatively with soil pH, and this can be explained considering that the repeated amendment with pomace determined in the 8y-A soil an increase in EC, mineral N, OC, and TN, compared to UA soil, but a decrease in soil pH. Following the same trend, MBC and fungal biomass was positively correlated with EC, mineral N, OC and TN and also each other, but negatively with pH; respiration was positively correlated with OC, TN, MBC and fungal biomass, and negatively with pH; qCO_2 was positively correlated with respiration and CEM. All these correlation better sensitized the results above explained.

Table 4.4 Pearson correlation coefficients calculated for soil chemical and biological parameters in UA and 8y-A control soils

	EC	Mineral N	OC	TN	C/N	MBC	qmic	F biomass	qfung	Cfung/Cmic	Respiration	qCO_2	CEM	AI3
pH	-0.823 ***	-0.805 ***	-0.821 ***	-0.820 ***	0.352	-0.794 ***	0.635 **	-0.716 ***	0.301	-0.326	-0.618 **	-0.301	0.485 *	0.746 ***
EC		0.943 ***	0.844 ***	0.866 ***	-0.433	0.846 ***	-0.69 **	0.818 ***	-0.056	0.485 *	0.426	-0.301	-0.741 ***	-0.901 ***
Mineral N			0.821 ***	0.905 ***	-0.482 *	0.770 ***	-0.767 ***	0.855 ***	-0.001	0.596 **	0.382	-0.299	-0.783 ***	-0.927 ***
OC				0.799 ***	-0.096	0.818 ***	-0.566 *	0.725 ***	-0.209	0.343	0.581 *	-0.136	-0.493 *	-0.796 ***
TN					-0.661 **	0.791 ***	-0.746 ***	0.854 ***	0.017	0.583 *	0.562 *	-0.130	-0.620 **	0.796 ***
C/N						-0.302	0.577 *	-0.521 *	-0.247	-0.552 *	-0.281	-0.046	0.345	0.516 *
MBC							-0.536 **	0.790 ***	-0.263	0.21	0.477 **	-0.318	-0.622 ***	-0.823 ***
qmic								0.777 ***	0.187	-0.662 ***	-0.680 ***	-0.303	0.313	0.856 ***
F biomass									0.170	0.757 ***	0.511 **	-0.139	-0.623 ***	-0.835 ***
qfung										0.596 ***	-0.379 *	-0.249	-0.187	0.271
Cfung /Cmic											0.290	0.090	-0.358	0.468 *
Respiration												0.660 ***	0.231	-0.543 **
qCO_2													0.799 ***	0.060
CEM														0.553 **

***= P<0.001; **= 0.001<P<0.01; *= 0.01<P<0.05, for chemical parameters n= 18; for biological parameters n=30

The potential beneficial or detrimental effects, on physical and chemical soil properties, derived by the agronomic use of olive pomace have been more studied compared to those regarding soil microbial community. The anti-microbial and toxic effects of olive mill wastes are well known (Paredes et al., 1987) and mainly ascribed to its phenolic fraction (Linares et al., 2003; Sampedro et al., 2008). The effects of agronomic use of olive-mill waste water have been largely studied in literature and widely reviewed by Barbera et al. (2013), highlighting that the direct spread on soils of olive-mill waste water could be limited by the oil and grease content, high salinity, acidity and phenolic compounds, that represent the main responsible for phytotoxic and antimicrobial effects. However little information are available on solid olive-mill wastes, and the differences in term of kind of solid olive-mill waste utilized in the experiments (due for example to the different oil extraction methods or to composting process), rate of applications, biological parameters assayed and also soil intrinsic characteristics make difficult the comparison among the results reported in literature.

The effects due to olive wastes on soil microbial community, in term of growth and activity changes, can arise upon two distinct effects: 1) the temporary enrichment of the soil with potentially available C sources and 2) the possible inhibitory effects of organic substances in the wastes towards microorganisms (Barbera et al., 2013); the global beneficial or detrimental impact on soil microbial community will be generated by the sum of these two effects. Therefore, it simply derives that the microbial response in term of biomass growth and activity is not unique.

For example, Sampedro et al. (2008) found that the application of dry solid olive-mill residues did not affect bacterial and fungal diversity (evaluated by 16S and 18S rDNA PCR-DGGE), but stimulated soil microbial activity at least at the levels applied, and increased the fungal/bacterial ratio. Similar results were also obtained by Giuntini et al. (2006), whose observed that amendment with either fresh or composted olive pomace had a limited impact on soil bacterial diversity; by contrast, they found marked differences in diversity of fungal community structure at

intermediate incubation times, suggesting that fungal community was affected by the organic amendments. Mechri et al. (2007) measured a marked increase in fungi in the short term after olive mill waste water application. However, in a specific study aiming to evaluate the potential use of olive pomace (from two-phase extraction system) and its compost as biodegradable pesticides against some fungi that are plant pathogens, Cayuela et al. (2008) observed in the laboratory bioassays a fungicidal property of the olive pomace extract (in particular against *Phytophthora capsici*, but not against *Pythium ultimum* and *Botrytis cinerea)*, but neither olive pomace nor compost extracts were able to inhibit the growth of the basidiomycetes *Rhizoctonia solani*.

An increase in microbial biomass C was observed by de La Fuente et al. (2011), who in a laboratory experiment found that both olive pomace and its compost increased the microbial biomass C and respiration compared to control, and in particular olive pomace treatment determined the highest microbial respiration and also the highest biomass C/biomass N ratio, and the latter effect was explained with a proliferation of fungi being the increase in this ratio related to the increase in fungal to bacterial biomass ratio. Moreover, the low concentration of water phenolic compounds measured in soil treated with pomace was index of no toxic effect. Clemente et al. (2007a) also found, in a field experiment, an increase in biomass C and biomass N, due to olive pomace application, only after two successive crop growths.

Nasini et al. (2013), in a field experiment regarding the same soil analysed in this study, after 4 years of amendment found very limited effects on soil microflora. Moreover, according to our study, Saviozzi et al. (2001) measured a general gradual increase of biomass C from the beginning of the experiment up to the 60^{th} day of incubation. Therefore as concerns the microbial biomass, our results are in agree with those found in literature, because we measured an increase in MB C due to pomace amendment in short term incubation, with more marked effects in the long term amendment that appeared comparing the two experimental conditions (8y-A and

UA); although fungal biomass was not affected by short term incubation, but only showed a significant increase after 8 years of pomace amendment, allowing to exclude negative or toxic effects on this component of soil microbial community.

In this study, soil respiration was stimulated both in the short term and in the long term by pomace amendment. In general the variation in respiration rate produced after organic amendment is explained by the "priming effect", described by Bingeman et al. (1953) as the short term change in the turnover of native soil organic matter, that can be shown after input of fresh organic matter and mineral fertilizer. It generally is temporary and lasts from several days to weeks, but a large amount of C and N may be released or immobilized in soil (Kuzyakov & Bol, 2006). Consequently, an increase in CO_2, CH_4, and N_2O emissions may occurs after amendment, due to extra decomposition of organic C and N compounds in soil stimulated by the fresh organic matter input. The causes of the "priming effect" are the changes in microbial activity and microbial growth (Blagodatskaya & Kuzyakov, 2008), that as consequence can increase the rate of SOM decomposition (De Nobili et al., 2001). In particular, the increase in CO_2 fluxes is largely caused by the changes in the microbial activity in response to altered amounts of utilizable C and N in soil (Perelo & Munch, 2005); in the specific case of organic amendment, the CO_2 emissions are part of a short-term C cycle and is not necessarily counted in greenhouse gasses emission. It has to be emphasized that a rapid but temporary increase in respiration and microbial biomass can be frequently observed also in response to olive mill waste water spreading (Kotsou et al., 2004; Saadi et al., 2007; Di Serio et al., 2008). The data of our study are in agreement with Saviozzi et al. (2001); in fact in their incubation experiment it found that in soil samples amended with moist olive pomace the evolution of CO_2-C increased, and also in this case the increase in CO_2 emission was larger in the initial phase, due to the rapid depletion of the easily mineralisable organic fractions, followed by a slower linear increase in time throughout the remaining period of incubation. However, our data are in contrast

with those reported by Ventorino et al. (2012), that in a field amendment with compost, found that soil microbial community was negatively affected by compost treatment, in term of microbial C, active fungal biomass, soil respiration and CEM, that appeared reduced after three years of compost application. On the contrary, we observed that, on the basis of the same parameters, the microbial community was not affected negatively by pomace, even if also in our study CEM was reduced by long term amendment with pomace. However the authors hypothesized that the effects reported in their study were due to the protection of organic matter from microbial attack operate both by humified mature compost (Spaccini et al., 2001; Piccolo et al., 2004) and by soil clay, that made organic compounds more resistant to microbial degradation, reducing the microbial mineralization capacity; they pointed out that longer period of observation generally are necessary to produce an improvement in soil biological quality.

Regarding the short term effect of olive mill waste water application on soil microbial community reported in literature, our observation were sometimes in agree and other ones in contrast. For example, contrary to us, Di Bene et al. (2013) found a microbial stress due to a soil disturbance after olive mill waste water field spread (at the maximum rate allowed by Italian law), underlined by a significant decrease in microbial biomass C and qmic, and a significant increase in soil respiration and metabolic quotient, even if the authors considered negligible this effects because no persisting in time (disappeared about after 6 months). A similar condition of stress was reported by Piotrowska et al. (2011), that in a laboratory incubation experiment in which the effects on soil of crude and de-oiled olive mill waste water were tested, observed, immediately after the beginning of incubation (two weeks), a strong increase in biomass C, qmic, soil respiration and qCO_2 in soil treated compared to control, assuming a minor stress in de-oiled olive waste water, probably due to a low amount of polyphenols than the crude one. Also in this case the impact of treatments

on soil microflora was explained by the temporary enrichment of the soil with readily available C source or the addition of inhibitory components to some microorganisms. The soil microbial indices could be useful tools in order to estimate the impacts of land use practices or soil managements, like the eco-physiological index that is metabolic quotient (qCO_2), based on physiological performances (respiration, C uptake, growth/death) on the total microbial biomass per unit time (Anderson & Domsch, 1989, Anderson, 2003). This approach derived from the application of the ecological theory of Odum (1969) on "*The Strategy of Ecosystem Development*" to soil microbial community: the development of diversity in ecosystems, for example a soil ecosystem, coincides with an increase of an efficient use of energy from a developmental to maturity and quasi-equilibrium stage, when a low community respiration per unit biomass is reached (Anderson & Domsch 2010). At equilibrium C inputs are equal to outputs, therefore no further accumulation or reduction of organic matter in that compartment can occur. Extrapolated to the soil microbial biomass compartment, this would mean that, at maturity, there should be a low microbial community respiration per unit of microbial biomass (qCO_2) and a high microbial biomass supported per unit energy source, which would be reflected in a high MBC/OC ratio (qmic). Moreover, the development of a more efficient microbial community may be related not only to the increasing diversity of organic matter input, but probably also to a greater species richness (Anderson & Domsch 2010). A high qCO_2 is sometimes explained with a high microbial activity and is interpreted as a positive property. In ecological terms, however, a high qCO_2 reflects a high maintenance carbon demand, and if the soil system cannot replenish the carbon which is lost through respiration, microbial biomass must decline (Anderson & Domsch 2010). Other important soil index is the microbial fraction of organic C (qmic), that can be used as a stability indicator for quick recognition of an environmental change; it indicates the contribution of microbial biomass to soil OC and also the substrate availability to the soil microbes (Anderson, 2003; Anderson & Domsch, 1989); in

general it give also information about the efficiency of conversion of the soil organic carbon into microbial C and therefore the fate of soil organic matter in specific conditions (stabilization *vs* losses). This two indices (i.e. qCO_2 and qmic) are considered good soil quality indicators, able to reflect the potential change after a cell impact (Anderson, 1994; Anderson & Domsch, 2010). A third microbial indicator is CEM (coefficient of endogenous mineralization) that represent the fraction of OC mineralized to CO_2 per unit of time, it is related to easily mineralisable OC amount in soil, because of the presence of more labile forms of OM produce an increase in mineralization rate. The CEM value can increase for example in response to soil addition of fresh organic material.

In our study, the laboratory treatments, in the short term, affected negatively the qmic, in fact lower value compared to control were observed in P-C and P-C+U for both soils and also for P+U in UA soil, therefore our results are in contrast with some literature findings; in fact different studies reported an increase in qmic in amended soil in the short period, such as in Di Bene et al. (2013) and Clemente et al. (2007a), an increase of this ratio was recorded, in the short term, after field spread of olive mill waste water. In our study, in the long term, 8y-A soil showed a lower value of qmic compared to UA soil, and also in this case the result contrasted with literature data. For example in long period amendment studies, Clemente et al. (2007a) and Lagomarsino et al. (2009) after one year from olive pomace amendment and 4 years from organic amendment, respectively, observed an increase in qmic. The low values of qmic that we recorded in the short or long term during the experiment could be explained with a low degradability of the organic matter provided to soil. In fact, in this case, the MBC after amendment increased, but organic C increased proportionally more than microbial biomass. In our study, no difference was observed for qCO_2 between the two soils and also among treatments. Also for this index, our results are in contrast with other results reported in literature, because after long-term organic amendment, generally a significant decrease in basal respiration per unit of

microbial biomass (qCO_2) was measured, as reported for example by Clemente et al. (2007a) after olive pomace amendment and by Lagomarsino et al. (2009) after organic amendment. In other studies it was reported that the soil application of olive mill waste water immediately increased the qCO_2, either in field and laboratory experiment (Di Bene et al., 2013; Piotrowska et al., 2011).

Synthesizing some literature findings, reduction in qCO_2 and increase in qmic were recorded after organic amendment that produced increase in soil organic C content (Lagomarsini et al., 2009; Clemente et al., 2007a and b), while generally increases in qCO_2 were joined sometime with an increase in microbial biomass and sometime with a reduction in qmic, as transient situation (for example immediately after olive waste application) with no correspondent variation in organic carbon content (Di Bene et al., 2013; Piotrowska et al., 2011), however both these situations were identified as stress conditions for soil microbial community.

In this work CEM was negatively affected by P-C+U treatment only in UA soil, where it was lower than in control, moreover CEM was also significantly lower in 8y-A soil compared to UA soil. The results for CEM index agree with those of Ventorino et al. (2012), that found a reduction in CEM following compost application. However, this result allows us to suppose that the provided organic amendment did not stimulate proportionally microbial activity, probably due to quality of organic matter, and thus can represent a lowly degradable organic substrate, able to increase in time soil carbon stock.

In the comparison between the types of soil, after 8 year of yearly olive pomace amendment a significant increase in OC was recorded associated also to an increase in soil microbial biomass and basal rate of respiration compared to UA soil, the microbial indices suggested that both microbial community appeared well adapted to respective condition (mature and with an efficient use of C source), because no differences in qCO_2 were observed. However the higher CEM value in UA soil indicate that in this experimental condition a more rapid mineralization rate with a

consequent major losses of OC occurs in unammended (UA) soil compared to 8y amended soil, where probably the repeated amendment caused an accumulation of organic C in more stable forms, suggesting also that in this condition the C reservoir is preserved. Moreover, in both soils the pomace treatment stimulated the growth (as MBC) and the activity (as respiration) of the soil microbial community in the short period, but this response in 8y-A soil was more marked than in UA soil, indicating that microbial community of 8y-A soil is better adapted to use pomace as organic source. No stress condition was identified in laboratory experiment as shown by no variation in qCO_2 index during the incubation and among soil experimental conditions.

The analyses of enzyme activities provided an additional information about the effects in short or long period of soil amendment. In fact it is well known that the activities of most enzymes increase as native SOM content increases, reflecting larger microbial communities and stabilization of enzymes on humic material (Bending et al., 2002). In literature different enzyme activities were measured in response to olive-mill residues application to soil to evaluate the effect in the short or long period. In general, in the short period, enzyme activities as dehydrogenase and fluorescein diacetate hydrolase appeared stimulated by different kinds of dry olive-mill residues (Sampedro et al., 2009), while in other cases, after olive-mill waste water soil application, no clear and evident trend of these enzyme activities was observed during the entire period of incubation, but a different behaviour was observed in relation to the kind of liquid by-product applied. For example, Piotrowska et al. (2011) did not observe differences in urease activity between control and de-oiled olive-mill waste water, while they observed a significant lower value after the addition of the crude one, probably due to the different chemical composition. Both García-Ruiz et al. (2012) and Lopéz Piñiero et al. (2011) in their studies on the long period effects of olive grove amendment with olive-mill residues (olive pomace compost and de-oiled two phase olive-mill waste) found 1) an increase in β-

glucosidase, explained as due to the acquisition of the capability by microbial community to utilize carbohydrate material added with the olive-mill residue and to the shift in the relative proportions of fungi and bacteria in soil, because this enzyme is produced by fungi; 2) an increase in soil phosphatase, which plays an essential role in the mineralization of organic phosphorus, explained by the fact that compost stimulates bacterial growth and enzyme production, including those involved in phosphorus and protein turnover; 3) an increase in urease activity compared to control. However, as effect in long term application, Lopéz Piñiero et al. (2011) observed that both phosphatase and urease showed a trend of reduction after many years of amendment, probably due to an increase in product concentration (available P and of NH_4^+, respectively) that produces a feedback inhibition (Criquet & Braud, 2008; García-Gil et al., 2000; Koning et al., 1966). Also Lagomarsino et al. (2009) observed a similar results in their study of field organic amendment for β-glucosidase, highlighting that may be a suitable indicator of change in C cycling, because indicates the SOM decomposition potential. The β-glucosidase has been proposed as sensitive index of management effects on agricultural soils (Ndiaye et al., 2000; De la Horra et al., 2003), suggesting that slightly increase of TOC can promote enzyme protection and induce significant changes of biochemical activity, indicating the efficacy of this enzyme to predict early changes in SOM content.

The enzyme activities essayed in this study showed a different behaviour in response to laboratory treatments in UA and 8y-A soils, in fact, while β-glucosidase was not affected by all treatments in both experimental conditions, urease was improved in UA soil by P treatment, and in 8y-A by P+U, P-C and P-C+U, and phosphatase increased in soil treated with P, P-C and P-C+U but only in UA condition; moreover all three activities were significantly enhanced by yearly pomace amendment (Fioretto et al., personal communication). These enzyme activities were utilised to calculate the index AI3, index developed by Puglisi et al. (2006) that was applied to three enzyme activities mostly frequently reported in literature. In the validation of

this index, the authors reported that in general altered soil had higher index scores than unaltered soils and it was able to discriminate between altered and unaltered soils under a wide range of conditions: irrigation with brackish water, heavy metal contamination, intensive agricultural exploitation, erosion. In our case clearly appeared that the yearly amendment caused an increase in soil quality, in fact AI3 index for 8y-A soil was significantly lower than UA soil, and in short period the index value decreased significantly compared to control in P+U, P-C and P-C+U treatments in 8y-A soil, condition in which the increase in urease enzyme activity was measured; however in literature other application of AI3 index on pomace amended soils were not found.

4.3.3. Short and long term effects of olive pomace amendment on soil N mineralization and nitrification

Ammonium is the preferred form in which microbes assimilate mineral N in many cultivated soil, while higher rate of NO_3^--N immobilization were found in forest or grassland (Stark & Hart, 1997; Hartch et al., 2000). The major fate of NH_4^+-N produced by mineralization in agricultural soil is nitrification (Robertson, 1997) and because NH_4^+-N is rapidly immobilized or nitrified, its concentration in soil is low and its residence time is short (<5 days; Murphy et al., 1998). Therefore, the measure of the amount of this two ions in soil give an indication about N dynamic and the main process involved.

The mineral N content as nitrate (NO_3^--N) and ammonium (NH_4^+-N) ions were measured at each time of sampling (Figure 4.3 a, b, c, d). Generally, both ions showed a variable trend during the incubation, in all treatments either in UA and 8y-A experimental condition. However the only relevant effect was found in UA soil P treatment in which always a low but significant decrease in NO_3^--N concentration compared to control was found (Figure 4.6 b) and sometimes compared also to other treatments. Similar results, during the entire period of observation, were also observed in 8y-A soil, but not every time (Figure 4.6 d). Moreover, higher values of

mineral N were found in 8y-A compared to UA soil, with a more marked effect for NO_3^--N ion.

Mineral N content measured on samples at 2 (T14) and 4 (T28) weeks after treatment application and on soil samples before the incubation (T0), was utilized to calculate N mineralization and N nitrification, applying the following equations:

$$N\ mineralization = \frac{(Mineral\ N)_F - (Mineral\ N)_I}{n}$$

$$Nitrification = \frac{(NO_3^- - N)_F - (NO_3^- - N)_I}{n}$$

where *Mineral N* is the sum of NO_3^--N and NH_4^+-N (µg N g^{-1} d.w.) and in particular *F* indicate their content at the end and *I* before the period of incubation; *n* indicate the time of incubation expressed in days (14 or 28 days).

The great variability in mineral N among all samples (as reported above), produced also a great variability in N mineralization and nitrification data (Figure 4.7), but with a differentiation between the two experimental conditions.

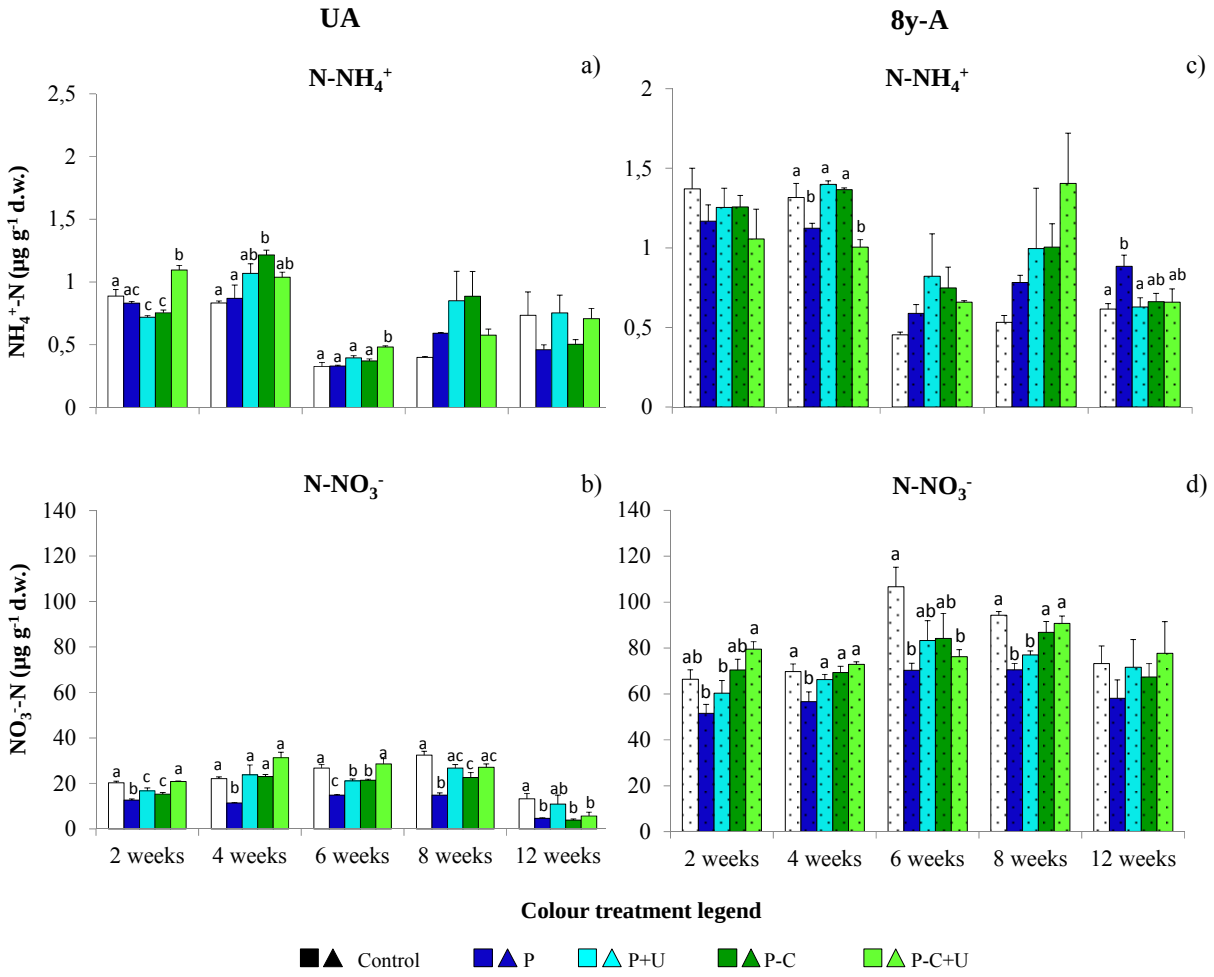


Figure 4.6 Mean values (+ standard errors) for ammonia (NH_4^+-N) and nitrate (NO_3^--N) ions concentrations in unamended soil (UA) in left side (a, b) and in field amended soil (8y-A) in right side (c, d), during the laboratory incubation experiment. Different letters indicate statistic differences among treatments ($P<0.05$).

The value of N mineralization rate (Figure 4.7 a) in UA soil at T14 appeared low either in untreated control and treated conditions, with no significant differences between treatments and control, while for nitrification at T14 (Figure 4.7 b) significant lower value were observed in UA soil for P and P-C treatments compared to control and other treatments. Similarly, at T28 in UA soil (Figure 4.7 c) the P+U and P-C+U treatments showed higher rates of N mineralization compared to other treatments; while at T28 in UA soil (Figure 4.7 d) nitrification showed a significant decrease in P treatment compared to control.

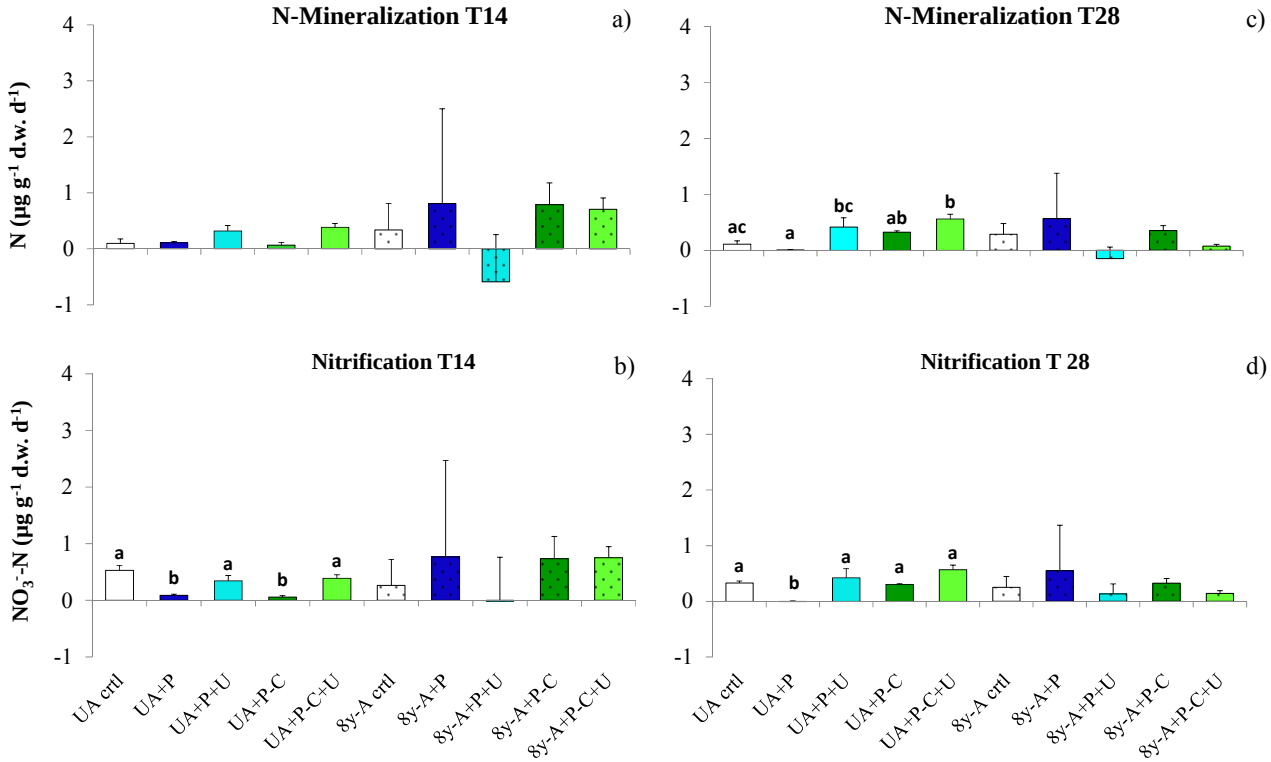


Figure 4.7 N mineralization and nitrification calculated for two sampling times, after 14 (T14) and 28 (T28) days after the beginning of incubation.

In 8y-A soil, a greater variability in N mineralization and nitrification data was record, thus both at T14 and at T28 days no clear trend was observable (Figure 4.7 a, b, c, d); however only in samples treated with P+U the activities appeared inhibited, even if no significant differences with control were measured. These results suggested a possible different behavior of both soils in response to laboratory treatments.

To evaluate the N mineralization and nitrification activities of both kinds of soils, following the specific method proposed by Castaldi et al (2009), a new incubation experiment was planned. Therefore both soils of the two experimental conditions (UA and 8y-A) were sampled twice from the same field plots, sieved in laboratory and utilized to prepare a new set of soil microcosms, assembled following the same procedure reported for the first experiment. The same treatments were tested, but this time each treatment was in five replicates. The microcosms were incubated (25°C, in the dark, 60% WHC) for two weeks, and at the end of this period, soil aliquots of each condition were extracted to measure the mineral N content, at time zero (T0), whereas another one was incubated twice at the same conditions in little jars for 14 days. At the end of incubation, NO_3^--N and NH_4^+-N ions were extracted from all samples (applying the procedure reported before) and after their potentiometric determination, the mineralization and nitrification rate were calculated. Moreover other soil aliquots of each sample were incubated for 21 days after substrate addiction (ammonium sulfate, 100 μg g^{-1}) to evaluate the potential nitrification, as well as the capacity of soil microbial community to conduct this reaction in not limited concentration of substrate. The NO_3^--N and NH_4^+-N content measured at T0 and T14 are reported in Figure 4.8, while the results of N mineralization, nitrification and potential nitrification are reported in Figure 4.9.

At T0 (i.e. at the end of period of incubation of soil microcosms) UA and 8y-A soils showed a significant lower value in NH_4^+-N in P-C+U treatment compared to control and also in P-C treatment but only for 8y-A soil (Figure 4.8 a). Moreover, the trend of

this parameter was different from the NH_4^+-N content showed before (Figure 4.6 a, c), where at each sampling times concentration of NH_4^+-N was higher in 8y-A soil than in UA soil. At T14, for UA soil, the amount of ammonium was reduced significantly compared to T0, but no difference was observed among treatment and control (Figure 4.8 c). In 8y-A soil a different behavior was observed among treatments: in fact the control at T14 showed the same content in NH_4^+-N of T0 (Figure 4.8 a, c), instead only P and P-C+U treatments reduced their amount compared to T0. The NO_3^--N content measured in this new experiment in both soils was higher compared to those in the first one, probably as a consequence of the different original two soil pools.

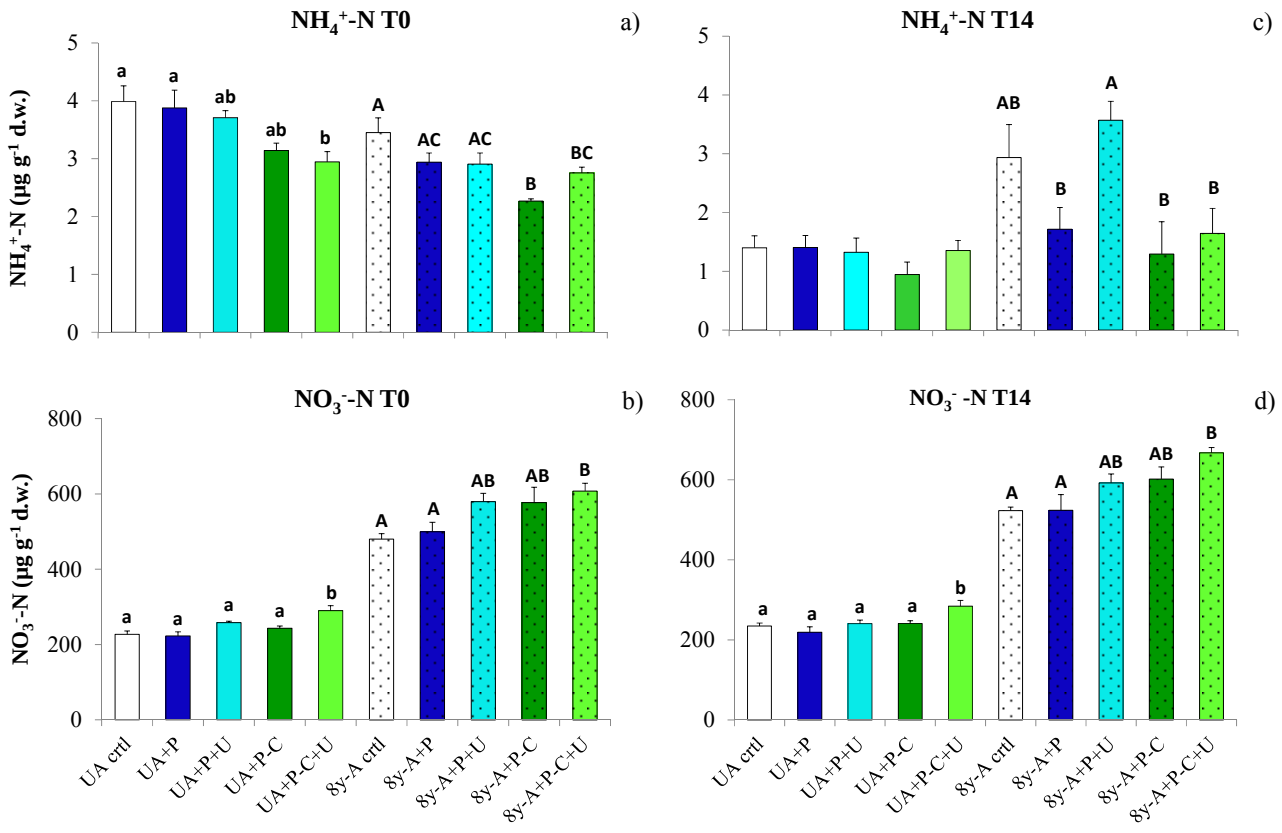


Figure 4.8 Mean (+ standard error) of the nitrate (NO_3^--N) and ammonia (NH_4^+-N) concentrations in unamended soil (UA) and in field amended soil (8y-A) before (T0) (left side, a, b) and after the incubation (T14) (right side c, d). Different letters indicate statistic differences among treatments ($P<0.05$) in each experimental condition.

At T0 and T14 days, the UA soil only showed higher content in P-C+U treated soil compared to control and other treatments (Figure 4.8 b and d), while 8y-A soil showed for the same treatment (P-C+U) a higher concentration of nitrate ion only compared to control and P treatment (Figure 4.8 d). However, in the same time NO_3^--N of 8y-A soil was higher compared to that of UA soil

In 8y-A soil, N mineralization and nitrification did not showed a clear trend among the treatments assayed (Figure 4.9 a, b, on the right side), even if a reduction (but not significant) could be observed after P and P+U treatments for both activities.

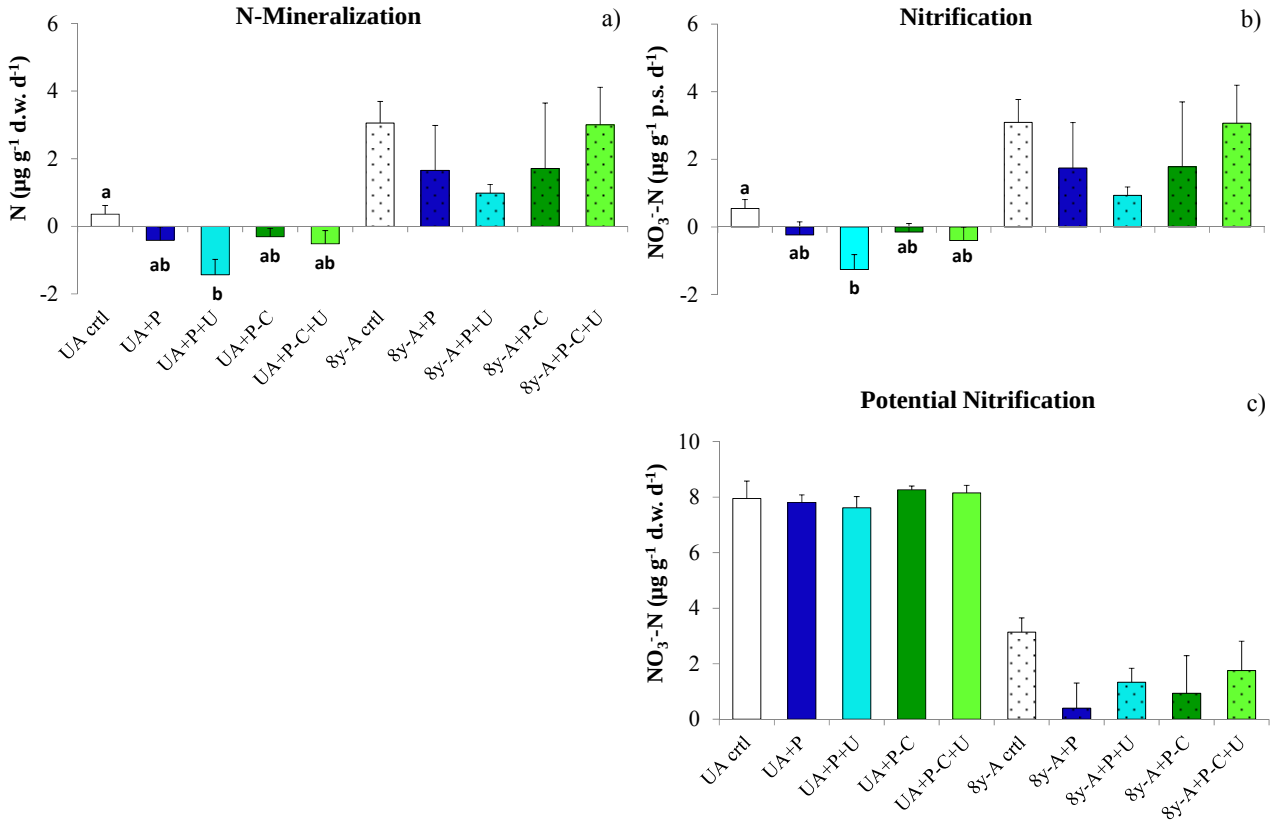


Figure 4.9 N mineralization (a), nitrification (b) and potential nitrification (c) calculated for the second incubation experiment. Different letters indicate statistic differences among treatments ($P<0.05$) in each experimental condition.

A similar effect was shown in UA soil, where N mineralization and nitrification showed negative values after all the treatments (Figure 4.8 a, b, on the left side), even if only for P+U condition the difference was significant (Figure 4.9 a, b). Really, it has to be emphasized that the trend in both soils (UA and 8y-A) resulted very similar, showing a decreased N mineralization and nitrification after pomace amendment, although this trend was more evident in UA soil because this soil had lower N mineralization and nitrification values compared to 8y-A soil (see controls for both soils in Figure 4.9 a, b), and the very low starting values of N mineralization and nitrification in UA soil probably caused the negative values of these activities after amendment addition. This effect, recorded in both soils, allowed us to hypothesized an inhibitory effect of pomace and composted pomace on N mineralization and nitrification processes in soils.

This hypothesis was further confirmed by the results of the potential nitrification activity (Figure 4.8 c). In fact, the data measured for this activity in studied soils (Figure 4.8 c) showed no effect due to treatments in both soils. Moreover, a surprising lower value of potential nitrification was found in 8y-A compared to UA soil. Theoretically, in condition of no limiting concentration of the substrate, as for potential nitrification, the nitrification activity may be enhanced. In our trial this effect was only observed for UA soil, where the rate of potential nitrification was higher than that of nitrification. On the contrary, in 8y-A soil the rate of nitrification was not improved by the substrate availability and no difference was observed between potential nitrification and nitrification (Figure 4.9 b, c). Therefore UA soil showed a higher rate in potential nitrification than 8y-A, while considering N mineralization and nitrification activities (i.e. in absence of the substrate) an opposite trend was shown.

All the results allowed us to suppose that N mineralization and nitrification processes was inhibited by addition of pomace in both soils, but this effect was emphasized by the prolonged amendment; in fact, after providing a mineral easily degradable

substrate, the nitrification resulted completely inhibited in 8y-A soil. Moreover, data from the two different incubation experiments showed no completely comparable results, effect probably due to the different sampling times, suggesting a different activity of microbial community involved in soil N cycle or different soil conditions that could influence the process.

Also in literature, contrasting results are reported about the effect of pomace or olive-pomace compost on N mineralization (Gòmez-Muños et al., 2011), because little information are available about the decomposition rate of pomace or its compost and the impact that these materials might have on the N availability in soils. For example some authors reported a significantly decrease in soil NO_3^--N and exchangeable NH_4^+ contents two years after olive pomace compost field application, hypothesizing N uptake (Montemurro et al., 2004); on the contrary Lòpez-Piñiero et al. (2008) observed an increase in concentration of inorganic N, as NO_3^--N, in soils since the second amendment with two phase olive mill waste, suggesting that mineral N had not been immobilized during the degradation of labile C pomace constituents. In our study, considering the long period amendment, we observed an increase in both N ions after 8 years, but no remarkable effect was due to organic amendment in the short period, with the except of a significant but low reduction of NO_3^--N in both soils amended with pomace.

Our results were also in agree with Gómez-Muñoz et al. (2011) and Cabrera et al. (2005) that evaluated the N mineralization in soil mixed with different types of olive pomace compost, founding no differences in inorganic nitrogen accumulation compared to control in treated soils, with an increasing trend in time towards negative values for the rates of N nitrification and N mineralization. Therefore Gómez-Muñoz et al. (2011) underlined that, despite the high total N content in the composts (about 2%) and the C/N ratio lower than 20, that was also our situation for both pomace and pomace-compost (Table 4.1), the expected net N mineralization did not occurred, with a net N immobilization; moreover, because of compost amendment did not

significantly increase the N_2O-N emissions, the losses from the incubated samples for denitrification and ammonia volatilization are also neglected (Gómez-Muñoz et al., 2011; Cabrera et al., 2005).

However in the comparison between N mineralization and nitrification between the two experimental conditions, our results are in agree with Cabrera et al. (2005) that found a N mineralization in amended soil that was higher than the control. Instead, considering the potential nitrification, that represented the most surprising result because clearly appeared a complete inhibition of the activity after long period amendment, our results contrasted with those of García-Ruiz et al. (2012), because in their study about the potential mineralized N in soil amended with pomace compost for different period, they found a significantly higher potential N nitrification in soils that received pomace compost annually for 9 to 16 years, whereas after a medium period of amendments (3 or 4 years) no differences existed between amended soils and control, affirming that nitrifying bacteria appeared stimulated only after long-term application of organic amendment.

Certainly more studied in literature are the effects of olive mill waste water on soil mineral N dynamic, but also in this case contrasting result are sometimes reported, for example Di Bene et al. (2013) measured temporary significant increase in NO_3^--N and NH_4^+-N content after olive-mill wastewater spread, that disappeared after six months, while in short period incubation Karpouzas et al. (2010) observed in a loamy sand soil amended with olive mill waste water a drastic reduction in NH_4^+-N and NO_3^- -N during the first 30 days of incubation compared to control, similarly to the lower content in nitrate measured in our soil in first incubation experiment after P addition for both UA and 8y-A soils. Moreover the authors also measured a significant change in community structure of ammonia-oxidizing bacteria in olive mil waste water amended soils. The ammonia-oxidizing bacteria, playing a critical role in N cycle, they are responsible for the first rate limiting step in nitrification in which ammonia (NH_3) is transformed to nitrate (NO_3^-) via nitrite (NO_2^-). This microflora

could be affected by a variety of chemical conditions including aromatic compounds, salts and soil pH. Moreover, nitrifying bacteria are pH sensitive, and generally the oxidation of ammonium-N is practically insignificant below the 5.6 threshold of pH while it is higher for pH values above 6.8 (Lang & Elliot, 1991), even if too high pH are also not favorable for some phylogenetic groups of nitrifying bacteria (Kowalchuk et al., 2000). For example, Mekki et al. (2006) observed that in field soil amended with a very high rate of biologically treated olive-mill waste water (400 m^3 ha^{-1}) the increase in soil pH up to 9.2 caused a significant reduction in the number of nitrifies (in term of CFU). However, the main a cause reported as responsible for the inhibition of N mineralization and potential nitrification activities, in response to olive-mill waste water application, are the polyphenols contained in these wastes, acting or by a selective inhibitory effect on soil nitrifying bacteria populations (Gamba et al., 2005; Di Serio et al., 2008), or by an ammonium immobilization, as reported by Aguilar (2010) that found a significant correlation between the amount of ammonium immobilized and polyphenols content in samples of olive-mill waste water. Aguilar (2010) found that olive-mill waste water was capable of blocking 25% of the initial N for pH values comprised between 5 and 12, supporting the idea that the application of an almost neutral solution of olive mill waste water on soil can reduce the nitrification. Moreover diluted olive-mill waste water in lightly acid pH was capable of retain ions nitrate in liquid complexes, that are not retained by the clay-humic complex of soil and are generally easily leached. Also Sierra et al. (2007) observed a temporary N immobilization after field application of olive mill waste water and Piotrowska et al. (2006) found a negative correlation between nitrate reductase and urea and between urease and extractable N in a short period incubation, supporting the idea that olive-mill waste water are able to immobilize the available N. Therefore the inhibition observed for olive-mill waste water was so pronounced that Aguilar (2010) proposed the use of this by-product as cheap nitrification inhibitor in order to reduce the nitrate contamination in groundwater.

It is conceivable that a similar effect could be occur also in pomace, equally rich in polyphenols, that can complex and immobilize the mineral N, inhibiting the potential nitrification in soil, and this effect could explain the inhibition of potential nitrification in 8y-A soil. Moreover, the not increase in NO_3^--N during the incubation could also be explained by the increase in microbial biomass occurred in both soils especially after treatment with P, that caused an increase in mineral N demand with an early immobilization of available NH_4^+-N in microbial biomass, reducing NH_4^+-N potentially convertible into NO_3^--N via nitrification with a consequent decline in levels of both ions (Karpouzas et al., 2010).

Globally, the observed effects could demonstrate that the organic N contained in pomace or pomace-compost is not ready available to medium timescale, and also suggests that most of organic N may be in relatively recalcitrant forms, and from pomace or pomace-compost agricultural applications can be expected an increase in organic matter and total N content in soils (Cabrera et al., 2005). In fact, because of in this study the mineral nitrogen constituted only a little portion of the TN in 8y-A soil (Table 4.2, 4.3) we can affirm that the most part of N in this soil is stored in organic form derived from the pomace yearly applied.

Concluding, our data suggested that an inorganic N immobilization occurred in soil after several years of amendment with pomace, probably due to its polyphenol content, that caused a reduced nitrification rate and thus also a reduced N mineralization, as clearly showed by inhibition effect measured in the 8 years amended soil on potential nitrification activity.

4.3.4. Short and long term effects of olive pomace amendment on soil C stock and its allocation in SOM fractions with different stability

The soil organic C stock in both UA and 8y-A soils was evaluated in bulk soil and in SOM fractions with different stability (Figure 4.10 a), by using control soils of the fourth and the fifth sampling.

The yearly soil amendment with olive pomace for 8 years, in a rate of 50 t ha^{-1}, caused a very significant increase in the organic C content in bulk soil, as showed previously in Table 4.3, that produced an increase in the relative organic C stock (calculated for unit of soil surface and for 10 cm of depth) amounting to +156%. This increase in OC stock was related to an increase in C content in all SOM fractions, with the highest percentage for the OC stock allocated in the light fraction (LF) (+503% in 8y-A compared to UA soil). This effect was expected because the organic amendment, by the addiction of fresh vegetable materials on the soil top layer, such as plants residues, causes firstly an increase in the more labile compounds. However, after 8 years of pomace amendment the OC stock increased in the more stable fractions of SOM too with a very high percentage: the OC stock of the 8y-A soil allocated in the hydrolysate fraction (HYD) was increased of +95% and that allocated in the recalcitrant fraction (REC) was increased of 106%, compared to UA soil. The increases in OC stock in bulk soil and in HYD and REC fractions of SOM were coupled with an increase in percent of modern C in the same fractions (pMC; Figure 4.7 b), and this result support the idea that the fresh organic carbon input, due to the pomace amendment, was also able to enrich the more stable fractions of SOM. In fact, the pMC of bulk soil and HYD fraction were very similar each other in both soils, but in 8y-A this values reached the horizontal line, indicating the limit of the total modern C. Also the REC fraction appeared enriched in ^{14}C in 8y-A compared to UA soil, showing that the OC stock of this fraction was really improved by recent OC inputs derived by the pomace amendment.

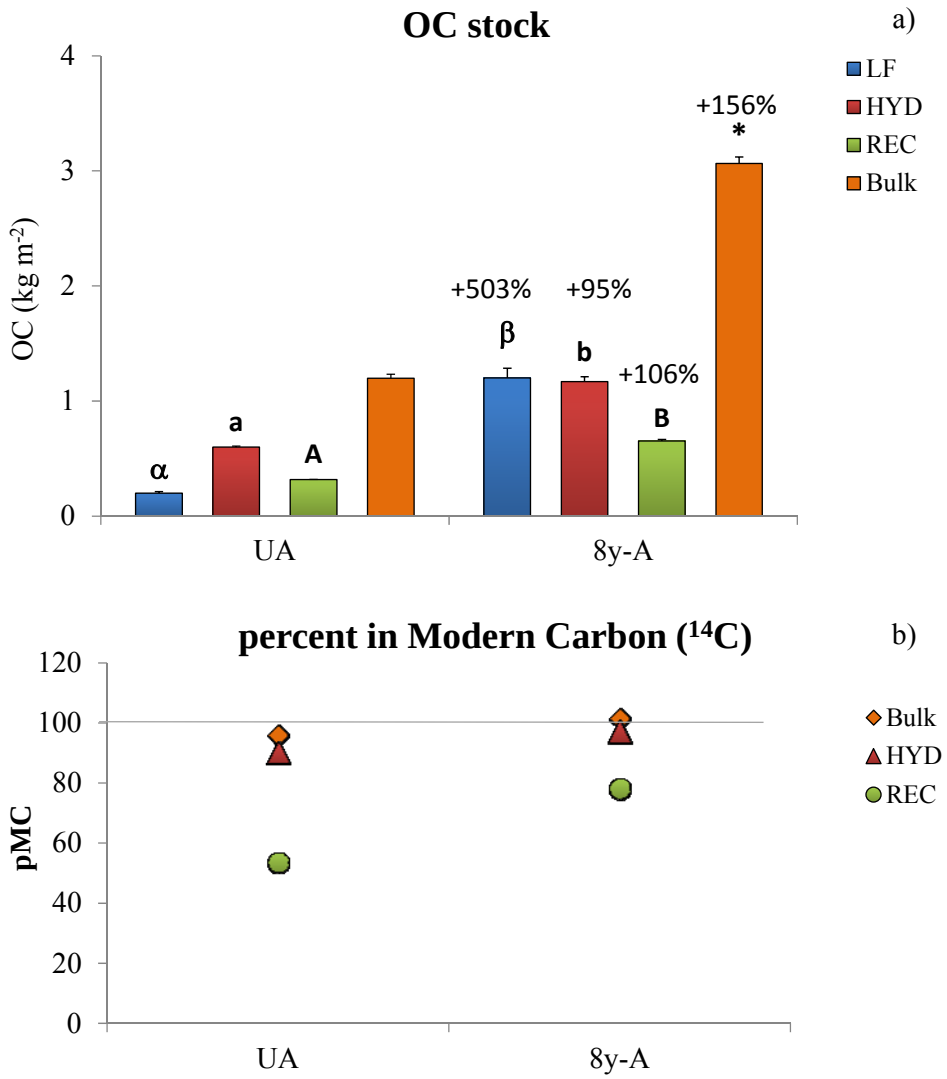


Figure 4.10 a): means values of organic C stocks (+ standard errors) in bulk soils (Bulk) and in SOM fractions with different stability (LF: light fraction; HYD: hydrolysable fraction; REC: recalcitrant fraction); b) percent in modern C (± relative error) in bulk soil and in HYD and REC fractions. UA: unamended soil; 8-yA: 8 year-amended soil. Different letters indicate significant differences between OC in each SOM fraction of 8-yA soil compared to that of UA soil (a specific font type was used for each SOM fraction); the asterisk indicates a significant difference in OC stock of bulk soil in the 8-yA compared to UA soil. On the bars is reported, for each fraction and bulk soil, the percentage of the increase measured in 8y- compared to UA soil.

The contribution in percentage of the OC_{REC} to total stock (Table 4.5) was similar in both soils (26.5% in UA and 21.6% in 8y-A) while great disparity appeared for both OC_{HYD} and OC_{LF}, in fact in UA soil OC_{HYD} and OC_{LF} stocks contribute for about 50.1% and 16.7%, respectively, while in 8y-A soil the two stocks had a similar percentage (38.1% for OC_{HYD} and 39.2% for OC_{LF}; see Table 4.5). Therefore in 8y-A soil the major part of the SOC stock is contained in the more labile pool (LF).

The amendment caused an improvement of the total OC stock that varied from 12.0 MgC ha^{-1} in UA soil to 30.6 MgC ha^{-1} in 8y-A soil, with a rate of increase in 8y-A soil that could be estimated in bulk soil of 2.3 MgC ha^{-1} y^{-1} and respectively in LF, HYD and REC fractions of 1.25, 0.71 and 0.41 MgC ha^{-1} y^{-1}. An increase in OC stock was also observed in the top 20 cm by Carbonell-Bojollo et al. (2009) after the application on soil of a two-phase olive-mill solid residue (from 16 to 21 MgC ha^{-1}) after three application of amendment, at the rate of 100 kg of solid residue for tree. However they also found an increase in deep horizons up to 60 cm; in fact, one year after the third yearly application, the total OC stock increased of 7.8 MgC ha^{-1} considering 60 cm of soil depth.

Table 4.5 Mean values (standard deviation) of the OC stock expressed in MgC ha^{-1} in bulk soil (Total SOC) and SOM fractions (OC_{LF}; OC_{HYD}; OC_{REC}) and their contribution in percentage to the total SOC stock. Asterisk indicate significant differences between the two experimental conditions.

	OC stocks (MgC ha^{-1})				% to Total SOC stock		
	Total SOC	OC_{LF}	OC_{HYD}	OC_{REC}	OC_{LF}	OC_{HYD}	OC_{REC}
UA	12.0 (±0.9)	2.0 (±0.3)	6.0 (±0.2)	3.2 (±0.1)	16.7	50.1	26.5
8y-A	30.6* (±1.4)	12.0* (±2.0)	11.7* (±1.1)	6.5* (±0.3)	39.2	38.1	21.3

Parras-Alcántara et al. (2013) also found a higher SOC stock in soil top layer in no-till (since 20 years) olive grove amended with manure, compared to an olive grove under conventional tillage; in particular, they measure an OC stock in the top 20 cm of conventional tilled olive grove of 22.54 MgC ha^{-1}, and in the top 20 cm of amended olive grove of 26.33 MgC ha^{-1}.

However in literature the distribution of OC stock among SOM fractions in organic amended agricultural soils is under investigated, and the study approach that we utilized never was applied before in a similar situation. The information that we derived from the measure about the OC stock in SOM fractions with different stability and about the ^{14}C content in the more stable fractions, highlighted that, despite the very high increase in OC stock in the more labile fraction, also the stocks in more stable fractions was improved, clearly confirmed by the enrichment in modern C of HYD and REC fractions. It means that the OC derived from this agronomic practices constituted a part of the OC stabilized in soil, in forms that will be preserved for a long time. However it is necessary to underline that the very high percentage of OC stock allocated in LF (39.2% in 8y-A *vs* 16.7% in UA), characterized also by the highest rate of accumulation, suggests that the olive pomace could be accumulates in soil in the more labile and active pool identifiable by LF, constituting an important reserve of OC for soil microbes and nutrients for plants.

4.4. Conclusion

The great variety in term of type of olive-mill residues potentially utilizable in agronomic applications, and also tested in laboratory or field experiment, make variable the effects observed in soils and very difficult to generalize the attended results, considering also that different properties of soils contribute to the variability of the results found in literature.

In this work, we aimed to provide a more clear and complete picture of the effects of olive pomace or composted olive pomace amendment on soil, in the short and long term, in order to define if this agronomic practice can be considered environmental safe and sustainable. For this purpose, we carried out a laboratory experiment, under controlled conditions, to test two soils from the same olive grove subjected to different management: a soil never amended with olive pomace and other one yearly amended for 8 years. A minimum data set of soil chemical and biological parameters useful to describe soil quality (including ecologically relevant microbial indices and the AI3 index) was assayed, deepening also some aspects of N cycle (such as N mineralization and nitrification). Moreover, organic C stock was quantified in bulk soil and in SOM fractions with different stability, by applying also the method of radiocarbon dating to better understand the fate of this organic amendment in soil.

Data showed that application of pomace and composted pomace determined, on the long term, an increase in total and mineral N and organic C, reducing also C/N ratio, but had also negative effects due to the reduction in soil pH and the increase in electrical conductivity. However, considering also the large set of the biological parameters assayed, we can affirm that the tested organic amendments did not cause toxic effects on soil microbial community, in the short or long term, but stimulated microbial growth and activity, with more marked effects in the long term, and generally determined beneficial effects on edaphic microflora, increasing soil quality. The AI3 index confirmed the improvement of soil quality due to the pomace amendment in the long term. As regards organic C stock, data of the microbial

indices (qCO_2, qmic and CEM) suggested that the organic C provided by pomace could be difficult to mineralize and thus was able to improve the organic C reservoir of agricultural soils. This hypothesis was further confirmed by the very significant increase in C stock, in the 8-years amended soil compared to unamended soil, that resulted allocated in stable fractions of SOM also. The results obtained by the radiocarbon dating method supported the idea that the fresh organic carbon input, due to the pomace amendment, was really able to enrich the more stable fractions of SOM. However, it has to be underlined that a very high percentage of the increased OC stock was allocated in the light fraction, the more labile and active C pool, constituting an important reserve of OC for soil microbes and nutrients for plants. Finally, the results of N mineralization and nitrification analyses showed that after 8 years of pomace amendment a reduction in soil of these activities occurred, suggesting a net N immobilization probably due to the high polyphenol content in the pomace, as also reported in literature.

Even if further studies are desirable to improve the knowledge on the sustainability of the agronomic use of olive mill wastes, in general, and olive pomace or pomace-compost, in particular, this study showed that olive pomace amendment can improve, in the long term, the soil quality and the stable C reservoir, constituting a good agricultural practice able to improve soils subjected to degradation for intensive agriculture, as well as agricultural soils of the Mediterranean area.

References

Aguilar, J.M. 2010. Fixation of ammonium-N and nitrate-N with olive oil mill astewaters. *Environmental Technology*, **31**(4), 395-8.

Alburquerque, J., Gonzálvez, J., García, D., Cegarra, J. 2007. Effects of a compost made from the solid by-product ("alperujo") of the two-phase centrifugation system for olive oil extraction and cotton gin waste on growth and nutrient content of ryegrass (Lolium perenne L.). *Bioresource Technology*, **98**(4), 940-945.

Alburquerque, J.A., Gonzalvez, J., Garcia, D., Cegarra, J. 2004. Agrochemical characterisation of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction. *Bioresour Technol*, **91**(2), 195-200.

Allen. 1989. Chemical analysis of ecological materials. *Blackwell Scientific Publication. Oxford.*

Amaral, C., Lucas, M.S., Coutinho, J., Crespí, A.L., do Rosário Anjos, M., Pais, C. 2008. Microbiological and physicochemical characterization of olive mill wastewaters from a continuous olive mill in Northeastern Portugal. *Bioresource Technology*, **99**(15), 7215-7223.

Anderson, J., Domsch, K. 1978. A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biology and Biochemistry*, **10**(3), 215-221.

Anderson, T.-H. 2003. Microbial eco-physiological indicators to asses soil quality. *Agriculture, ecosystems & environment*, **98**(1), 285-293.

Anderson, T.-H., Domsch, K.H. 1989. Ratios of microbial biomass carbon to total organic carbon in arable soils. *Soil Biology and Biochemistry*, **21**(4), 471-479.

Anderson, T.-H., Domsch, K.H. 2010. Soil microbial biomass: the eco-physiological approach. *Soil Biology and Biochemistry*, **42**(12), 2039-2043.

Anderson, T. 1994. Physiological analysis of microbial communities in soil: applications and limitations. In: Ritz, K., Dighton, J., Giller, K.E. (Eds), *Beyoond the Biomass*. Wiley, Chichester, pp. 67-76.

Antolín, M.C., Pascual, I., García, C., Polo, A., Sánchez-Díaz, M. 2005. Growth, yield and solute content of barley in soils treated with sewage sludge under semiarid Mediterranean conditions. *Field Crops Research*, **94**(2–3), 224-237.

Barbera, A., Maucieri, C., Cavallaro, V., Ioppolo, A., Spagna, G. 2013. Effects of spreading olive mill wastewater on soil properties and crops, a review. *Agricultural Water Management*, **119**, 43-53.

Bending, G.D., Turner, M.K., Jones, J.E. 2002. Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. *Soil Biology and Biochemistry*, **34**(8), 1073-1082.

Berg, B., Wessèn, B., 1984. Changes in organic-chemical components and in growth of fungal mycelium in decomposing birch leaf as campared to pine needles. *Pedobiologia,* **26**: 258-296.

Bertrand, J.-C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., Sime-Ngando, T. 2015. Environmental microbiology: Fundamentals and applications. Springer, pp. 578.

Bingeman, C.W., Varner, J., Martin, W. 1953. The effect of the addition of organic materials on the decomposition of an organic soil. *Soil Science Society of America Journal*, **17**(1), 34-38.

Blagodatskaya, E., Kuzyakov, Y. 2008. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. *Biology and Fertility of Soils*, **45**(2), 115-131.

Bonanomi, G., D'Ascoli, R., Scotti, R., Gaglione, S.A., Caceres, M.G., Sultana, S., Scelza, R., Rao, M.A., Zoina, A. 2014. Soil quality recovery and crop yield enhancement by combined application of compost and wood to vegetables grown under plastic tunnels. *Agriculture, ecosystems & environment*, **192**, 1-7.

Brunetti, G., Plaza, C., Senesi, N. 2005. Olive pomace amendment in Mediterranean conditions: effect on soil and humic acid properties and wheat (Triticum turgidum L.) yield. *Journal of Agricultural anf Food Chemistry*, **53**(17), 6730-7.

Cabrera, F., Martín-Olmedo, P., López, R., Murillo, JM., 2005. Nitrogen mineralization in soils amended with composted olive mill sludge. *Nutrient Cycling in Agroecosystems*, **71**, 249–258.

Camposeo, S., Vivaldi, G.A. 2011. Short-term effects of de-oiled olive pomace mulching application on a young super high-density olive orchard. *Scientia Horticulturae*, **129**(4), 613-621.

Caputo, A.C., Scacchia, F., Pelagagge, P.M. 2003. Disposal of by-products in olive oil industry: waste-to-energy solutions. *Applied Thermal Engineering*, **23**(2), 197-214.

Carbonell-Bojollo, R., Ordóñez-Fernández, R., Rodríguez-Lizana, A. 2009. Influence of olive mill waste application on the role of soil as a carbon source or sink. *Climatic Change*, **102**(3), 625-640.

Castaldi, S., Aragosa, D. 2002. Factors influencing nitrification and denitrification variability in a natural and fire-disturbed Mediterranean shrubland. *Biology and Fertility of Soils*, **36**(6), 418-425.

Castaldi, S., Carfora, A., Fiorentino, A., Natale, A., Messere, A., Miglietta, F., Cotrufo, M.F. 2009. Inhibition of net nitrification activity in a Mediterranean

woodland: possible role of chemicals produced by Arbutus unedo. *Plant and soil*, **315**(1-2), 273-283.

Castanha, C., Trumbore, S., Amundson, R. 2008b. *Methods of Separating Soil Carbon Pools Affect the Chemistry and Turnover Time of Isolated Fractions*.

Cayuela, M., Millner, P., Meyer, S., Roig, A. 2008. Potential of olive mill waste and compost as biobased pesticides against weeds, fungi, and nematodes. *Science of The Total Environment*, **399**(1), 11-18.

Chartzoulakis, K., Psarras, G., Moutsopoulou, M., Stefanoudaki, E. 2010. Application of olive mill wastewater to a Cretan olive orchard: Effects on soil properties, plant performance and the environment. *Agriculture, ecosystems & environment*, **138**(3), 293-298.

Chowdhury, A.K.M.M.B., Akratos, C.S., Vayenas, D.V., Pavlou, S. 2013. Olive mill waste composting: A review. *International biodeterioration & biodegradation*, **85**, 108-119.

Clemente, R., De la Fuente, C., Moral, R., Bernal, M. 2007a. Changes in microbial biomass parameters of a heavy metal-contaminated calcareous soil during a field remediation experiment. *Journal of environmental quality*, **36**(4), 1137-1144.

Clemente, R., Paredes, C., Bernal, M. 2007b. A field experiment investigating the effects of olive husk and cow manure on heavy metal availability in a contaminated calcareous soil from Murcia (Spain). *Agriculture, ecosystems & environment*, **118**(1), 319-326.

Criquet, S., Braud, A. 2008. Effects of organic and mineral amendments on available P and phosphatase activities in a degraded Mediterranean soil under short-term incubation experiment. *Soil and Tillage Research*, **98**(2), 164-174.

D'Ascoli, R., Rutigliano, F.A., De Pascale, R.A., Gentile, A., De Santo, A.V. 2005. Functional diversity of the microbial community in Mediterranean maquis soils as affected by fires. *International Journal of Wildland Fire*, **14**(4), 355-363.

de la Fuente, C., Clemente, R., Martínez-Alcalá, I., Tortosa, G., Bernal, M.P. 2011. Impact of fresh and composted solid olive husk and their water-soluble fractions on soil heavy metal fractionation; microbial biomass and plant uptake. *Journal of hazardous materials*, **186**(2), 1283-1289.

De la Horra, A., Conti, M., Palma, R. 2003. β-Glucosidase and Proteases Activities as Affected by Long-Term Management Practices in a Typic Argiudoll Soil. *Communications in soil science and plant analysis*, **34**(17-18), 2395-2404.

De Nobili, M., Contin, M., Mondini, C., Brookes, P. 2001. Soil microbial biomass is triggered into activity by trace amounts of substrate. *Soil Biology and Biochemistry*, **33**(9), 1163-1170.

Di Bene, C., Pellegrino, E., Debolini, M., Silvestri, N., Bonari, E. 2013. Short-and long-term effects of olive mill wastewater land spreading on soil chemical and biological properties. *Soil Biology and Biochemistry*, **56**, 21-30.

Di Serio, M., Lanza, B., Mucciarella, M., Russi, F., Iannucci, E., Marfisi, P., Madeo, A. 2008. Effects of olive mill wastewater spreading on the physico-chemical and microbiological characteristics of soil. *International biodeterioration & biodegradation*, **62**(4), 403-407.

Dilly, O., Munch, J.C. 1998. Ratios between estimates of microbial biomass content and microbial activity in soils. *Biology and Fertility of Soils*, **27**(4), 374-379.

Eivazi, F., Tabatabai, M. 1988. Glucosidases and galactosidases in soils. *Soil Biology and Biochemistry*, **20**(5), 601-606.

Eivazi, F., Tabatabai, M. 1977. Phosphatases in soils. *Soil Biology and Biochemistry*, **9**(3), 167-172.

Eurostat. 2008. Agricultural Statistics. Main Results – 2006–2007. *Office for Official Publications of the European Communities, Luxemburg.*

Fernández-Hernández, A., Roig, A., Serramiá, N., Civantos, C.G.-O., Sánchez-Monedero, M.A. 2014. Application of compost of two-phase olive mill waste on olive grove: Effects on soil, olive fruit and olive oil quality. *Waste Management*, **34**(7), 1139-1147.

Fleskens, L., Stroosnijder, L. 2007. Is soil erosion in olive groves as bad as often claimed? *Geoderma*, **141**(3–4), 260-271.

Gamba, C., Piovanelli, C., Papini, R., Pezzarossa, B., Ceccarini, L., Bonari, E., 2008. Soil microbial characteristics and mineral nitrogen availability as affected by olive oil waste water applied to cultivated soil. *Communications Soil Science and Plant Analyses*, **36**, 937–950.

Garcıa-Gil, J., Plaza, C., Soler-Rovira, P., Polo, A. 2000. Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass. *Soil Biology and Biochemistry*, **32**(13), 1907-1913.

García-Ruiz, R., Ochoa, M.V., Hinojosa, M.B., Gómez-Muñoz, B. 2012. Improved soil quality after 16 years of olive mill pomace application in olive oil groves. *Agronomy for sustainable development*, **32**(3), 803-810.

Gijsman, A.J., Alarcón, H., Thomas, R.J. 1997. Root decomposition in tropical grasses and legumes, as affected by soil texture and season. *Soil Biology and Biochemistry*, **29**(9), 1443-1450.

Giuntini, E., Bazzicalupo, M., Castaldini, M., Fabiani, A., Miclaus, N., Piccolo, R., Ranalli, G., Santomassimo, F., Zanobini, S., Mengoni, A. 2006. Genetic diversity of

dinitrogen-fixing bacterial communities in soil amended with olive husks. *Annals of microbiology*, **56**(2), 83-88.

Gómez-Muñoz, B., Hatch, D.J., Bol, R., Dixon, E.R., García-Ruiz, R. 2011. Gross and net rates of nitrogen mineralisation in soil amended with composted olive mill pomace. *Rapid Communications in Mass Spectrometry*, **25**(11), 1472-1478.

Harris, D., Horwáth, W.R., van Kessel, C. 2001. Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis. *Soil Science Society of America Journal*, **65**(6), 1853-1856.

Hartch, D.J., Jarvis, S.C., Parkinson, R.J., Lovell, R.D., 2000. Combining field incubation with nitrogen-15 labelling to examine nitrogen trasformations in low to high intensity grassland management systems. Biology and Fertility of Soil, 30, 492-499.

Jenkinson, D., Davidson, S., Powlson, D. 1979. Adenosine triphosphate and microbial biomass in soil. *Soil Biology and Biochemistry*, **11**(5), 521-527.

Kandeler, E., Gerber, H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biology and Fertility of Soils*, **6**(1), 68-72.

Karpouzas, D.G., Ntougias, S., Iskidou, I., Rousidou, C., Papadopoulou, K.K., Zervakis, G.I., Ehaliotis, C., 2010. Olive mill wastewater affects the structure of soil bacterial communities. *Applied Soil Ecology*, **45**, 101–111.

Killham, K. 1994. Soil ecology. Cambridge University Press.

Kieft, T.L., White, C.S., Loftin, S.R., Aguilar, R., Craig, J.A., Skaar, D.A. 1998. Temporal dynamics in soil carbon and nitrogen resources at a grassland-shrubland ecotone. *Ecology*, **79**(2), 671-683.

Konig, C., Kaltwasser, H., Schiegel, H. 1966. The formation of urease after use of other nitrogen sources in Hydrogenomonas. *Archives of Microbiology*, **53**, 231-241.

Kotsou, M., Mari, I., Lasaridi, K., Chatzipavlidis, I., Balis, C., Kyriacou, A. 2004. The effect of olive oil mill wastewater (OMW) on soil microbial communities and suppressiveness against Rhizoctonia solani. *Applied Soil Ecology*, **26**(2), 113-121.

Kowalchuk, G.A., Stienstra, A.W., Heilig, G.H., Stephen, J.R., Woldendorp, J.W., 2000. Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environmental Microbiology*, **2**, 99–110.

Kuzyakov, Y., Bol, R. 2006. Sources and mechanisms of priming effect induced in two grassland soils amended with slurry and sugar. *Soil Biology and Biochemistry*, **38**(4), 747-758.

Lagomarsino, A., Moscatelli, M., Di Tizio, A., Mancinelli, R., Grego, S., Marinari, S. 2009. Soil biochemical indicators as a tool to assess the short-term impact of agricultural management on changes in organic C in a Mediterranean environment. *Ecological Indicators*, **9**(3), 518-527.

Lang, H., Elliot, G., 1991. Influence of Ammonium: Nitrate ratio and nitrogen concentration on nitrification activity in soilless potting media*, Journal of the American Society for Horticultural Science,* **116**, 642–645.

Linares, A., Manuel Caba, J., Ligero, F., de la Rubia, T., Martínez, J. 2003. Detoxification of semisolid olive-mill wastes and pine-chip mixtures using Phanerochaete flavido-alba. *Chemosphere*, **51**(9), 887-891.

Lopez-Pineiro, A., Albarran, A., Nunes, J.M., Barreto, C. 2008. Short and medium-term effects of two-phase olive mill waste application on olive grove production and

soil properties under semiarid mediterranean conditions. *Bioresource Technology*, **99**(17), 7982-7.

López-Piñeiro, A., Albarrán, A., Rato Nunes, J.M., Peña, D., Cabrera, D. 2011. Long-term impacts of de-oiled two-phase olive mill waste on soil chemical properties, enzyme activities and productivity in an olive grove. *Soil and Tillage Research*, **114**(2), 175-182.

López-Piñeiro, A., Silva, C.d., Nunes, J., Rozas, M., García Navarro, A., Rubio, J., Morgan, R., Asins, S., Andreu, V. 2002. Effects of the solid olive mills waste application on soil properties of irrigated olive grove. *Man and soil at the Third Millennium. Proceedings International Congress of the European Society for Soil Conservation, Valencia, Spain, 28 March-1 April, 2000. Volume 2*. GEOFORMA Edicions, SL. pp. 1117-1122.

Lozano-García, B., Parras-Alcántara, L. 2013. Short-term effects of olive mill by-products on soil organic carbon, total N, C:N ratio and stratification ratios in a Mediterranean olive grove. *Agriculture, ecosystems & environment*, **165**, 68-73.

Mahmoud, M., Janssen, M., Haboub, N., Nassour, A., Lennartz, B. 2010. The impact of olive mill wastewater application on flow and transport properties in soils. *Soil and Tillage Research*, **107**(1), 36-41.

Marzaioli, F., Lubritto, C., Del Galdo, I., D'Onofrio, A., Cotrufo, M.F., Terrasi, F., 2010. Comparison of different soil organic matter fractionation methodologies: evidences from ultrasensitive 14C measurements. *Nuclear Instruments and Methods in Physics Research B*, **268**, 1062-1066.

Marzaioli, R., D'Ascoli, R., De Pascale, R.A., Rutigliano, F.A. 2010. Soil quality in a Mediterranean area of Southern Italy as related to different land use types. *Applied Soil Ecology*, **44**(3), 205-212.

Mechri, B., Echbili, A., Issaoui, M., Braham, M., Elhadj, S.B., Hammami, M. 2007. Short-term effects in soil microbial community following agronomic application of olive mill wastewaters in a field of olive trees. *Applied Soil Ecology*, **36**(2), 216-223.

Mekki, A., Dhouib, A., Sayadi, S., 2006. Changes in microbial and soil properties

following amendment with treated and untreated olive mill wastewater. *Microbiological Research*, **161**, 93–101.

Mekki, A., Dhouib, A., Sayadi, S. 2009. Evolution of several soil properties following amendment with olive mill wastewater. *Progress in Natural Science*, **19**(11), 1515-1521.

Montemurro, F., Convertini, G., Ferri, D. 2004. Mill wastewater and olive pomace compost as amendments for rye-grass. *Agronomie*, **24**(8), 481-486.

Murphy, D.V., Fillery, R.P., Sparling, G.P., 1998. Seasonal fluctuations in gross N mineralisation, ammonium consumption, and microbial biomass in a Western Australian soil under different land uses. *Australian Journal of Agricultural Research*, **49**, 523-535.

Nasini, L., Gigliotti, G., Balduccini, M.A., Federici, E., Cenci, G., Proietti, P. 2013. Effect of solid olive-mill waste amendment on soil fertility and olive (Olea europaea L.) tree activity. *Agriculture, ecosystems & environment*, **164**, 292-297.

Ndiaye, E., Sandeno, J., McGrath, D., Dick, R. 2000. Integrative biological indicators for detecting change in soil quality. *American Journal of Alternative Agriculture*, **15**(1), 26-36.

Netti, S., Wlassics, I. 1995. Studio sulle metodologie di smaltimento delle acque di vegetazione. *Rivista Italiana delle Sostanze Grasse*, **72**(3), 119-125.

Niaounakis, M., Halvadakis, C.P. 2006. Olive Processing Waste Management: Literature Review and Patent Survey 2nd Edition. Elsevier.

Odum, E.P. 1969. The strategy of ecosystem development. *Sustainability: Sustainability*, **164**, 58.

Olson, F. 1950. Quantitative estimates of filamentous algae. *Transactions of the American Microscopical Society*, **69**(3), 272-279.

Ouzounidou, G., Zervakis, G.I., Gaitis, F. 2010. Raw and microbiologically detoxified olive mill waste and their impact on plant growth. *Terrestrial and Aquatic Environmental Toxicology*, **4**(1), 21-38.

Owen, R.W., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B., Bartsch, H. 2000. Identification of lignans as major components in the phenolic fraction of olive oil. *Clinical Chemistry*, **46**(7), 976-88.

Paredes, C., Bernal, M., Cegarra, J., Roig, A. 2002. Bio-degradation of olive mill wastewater sludge by its co-composting with agricultural wastes. *Bioresource Technology*, **85**(1), 1-8.

Paredes, C., Cegarra, J., Bernal, M.P., Roig, A. 2005. Influence of olive mill wastewater in composting and impact of the compost on a Swiss chard crop and soil properties. *Environment International*, **31**(2), 305-12.

Paredes, M.J., Moreno, E., Ramos-Cormenzana, A., Martinez, J. 1987. Characteristics of soil after pollution with waste waters from olive oil extraction plants. *Chemosphere*, **16**(7), 1557-1564.

Parras-Alcántara, L., Díaz-Jaimes, L., Lozano García, B., 2013. organic Farming affects C and N in soil under ilive groves in Mediterranean areas. Land Degradation & Development, **26**, 800-806.

Perelo, L.W., Munch, J.C. 2005. Microbial immobilisation and turnover of 13 C labelled substrates in two arable soils under field and laboratory conditions. *Soil Biology and Biochemistry*, **37**(12), 2263-2272.

Piccolo, A., Spaccini, R., Nieder, R., Richter, J. 2004. Sequestration of a biologically labile organic carbon in soils by humified organic matter. *Climatic Change*, **67**(2-3), 329-343.

Piotrowska, A., Iamarino, G., Rao, M.A., Gianfreda, L., 2006. Short-term effects of olive mill waste water (OMW) on chemical and biochemical properties of a semiarid Mediterranean soil. *Soil Biology & Biochemistry* **38**, 600–610.

Piotrowska, A., Rao, M.A., Scotti, R., Gianfreda, L. 2011. Changes in soil chemical and biochemical properties following amendment with crude and dephenolized olive mill waste water (OMW). *Geoderma*, **161**(1), 8-17.

Plaza, C., Senesi, N., Brunetti, G., Mondelli, D., 2007. Evolution of the fulvic acid fractions during co-composting of olive mill wastewater sludge and tree cuttings. *Bioresources and Technology*, **98**:1964–1971.

Puglisi, E., Del Re, A., Rao, M., Gianfreda, L. 2006. Development and validation of numerical indexes integrating enzyme activities of soils. *Soil Biology and Biochemistry*, **38**(7), 1673-1681.

Robertson, G.P., 1997. Nitrogen use efficiency in row crop agricolture: crop nitrogen use and soil nitrogen loss. In Jackson, L., (Ed), *Ecology in Agriculture, Academic Press*, New York, pp. 347-365.

Rodis, P.S., Karathanos, V.T., Mantzavinou, A. 2002. Partitioning of olive oil antioxidants between oil and water phases. *Journal of Agricultural and Food Chemistry*, **50**(3), 596-601.

Roig, A., Cayuela, M.L., Sanchez-Monedero, M.A. 2006. An overview on olive mill wastes and their valorisation methods. *Waste Management*, **26**(9), 960-9.

Rozzi, A., Malpei, F. 1996. Treatment and disposal of olive mill effluents. *International biodeterioration & biodegradation*, **38**(3), 135-144.

Rutigliano, F.A., D'Ascoli, R., Virzo De Santo, A., 2004. Soil microbial metabolism and nutrient status in a Mediterranean area as affected by plant cover. Soil Biology and Biochemistry **36**, 1719–1729.

Saadi, I., Laor, Y., Raviv, M., Medina, S. 2007. Land spreading of olive mill wastewater: effects on soil microbial activity and potential phytotoxicity. *Chemosphere*, **66**(1), 75-83.

Sampedro, I., Giubilei, M., Cajthaml, T., Federici, E., Federici, F., Petruccioli, M., D'annibale, A. 2009. Short-term impact of dry olive mill residue addition to soil on the resident microbiota. *Bioresource Technology*, **100**(23), 6098-6106.

Sampedro, I., Marinari, S., Ocampo, J., García-Romera, I., Petruccioli, M., Federici, F., D'Annibale, A. 2008. The use of immobilized white-rot fungi reduce the time requirements and improves detoxification of two-phase dry olive mill residue. *Journal of Agricultural and Food Chemistry*, **57**, 5452-5460.

Saviozzi, A., Levi-Minzi, R., Cardelli, R., Biasci, A., Riffaldi, R. 2001. Suitability of moist olive pomace as soil amendment. *Water, Air, and Soil Pollution*, **128**(1-2), 13-22.

Scotti, R., D'Ascoli, R., Gonzalez Caceres, M., Bonanomi, G., Sultana, S., Cozzolino, L., Scelza, R., Zoina, A., Rao, M.A. 2015. Combined use of compost and wood scraps to increase carbon stock and improve soil quality in intensive farming systems. *European Journal of Soil Science*, **66**(3), 463-475.

Sierra, J., Martí, E., Garau, M.A., Cruañas, R. 2007. Effects of the agronomic use of olive oil mill wastewater: Field experiment. *Science of The Total Environment*, **378**(1–2), 90-94.

Soil Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. *United States Departmentof Agriculture and Natural Resources Conservation Service, Washington, DC.*

Spaccini, R., Zena, A., Igwe, C., Mbagwu, J., Piccolo, A. 2001. Carbohydrates in water-stable aggregates and particle size fractions of forested and cultivated soils in two contrasting tropical ecosystems. *Biogeochemistry*, **53**(1), 1-22.

Strak, J.M., Hart, S.C., 1997. High rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature,* **385**, 61-64

Sundman, V., Sivela, S., 1978. A comment on the membrane filter technique for estimation of length of fungal hyphae in soil. *Soil Biology & Biochemistry,* **10**, 399–401.

Swift, M.J., Heal, O.W., Anderson, J.M., 1979. Decomposition in terrestrial ecosystems. University of California Press, Berkeley.

Tisdale, S., Nelson, W., Havlin, J., Beaton, J., 1999. Soil fertility and fertilizers. In: An Introduction to Nutrient Management, 6th ed. Prentice-Hall, Upper Saddlen River, New Jersey, pp. 406–425.

Trumbore, S.E., Zheng, S. 1996. Comparison of fractionation methods for soil organic matter 14C analysis. *Radiocarbon*, **38**(2), 219-229.

U.S.D.A. 1993. Soil Survey Division Staff, Soil Survey Manual, Soil Conservation Servise. *Handbook* **18**.

Ventorino, V., De Marco, A., Pepe, O., De Santo, A.V., Moschetti, G. 2012. Impact of innovative agricultural practices of carbon sequestration on soil microbial community. In Piccolo, A. (Ed): *Carbon Sequestration in Agricultural Soils*, Springer, pp. 145-177.

Vitolo, S., Petarca, L., Bresci, B. 1999. Treatment of olive oil industry wastes. *Bioresource Technology*, **67**(2), 129-137.

Wardle, D., Ghani, A. 1995. A critique of the microbial metabolic quotient (qCO 2) as a bioindicator of disturbance and ecosystem development. *Soil Biology and Biochemistry*, **27**(12), 1601-1610.

Chapter 5- Organic carbon stability and distribution in soil macro- and micro-aggregates of three Chilean Andisols

5.1. Introduction

Andic soils are typical soils developed from volcanic ash and it has been evaluated that they store about the 5% of the global soil C (Eswaran et al., 1993). These soils are characterized by a high soil organic matter (SOM) content, good physical properties, and a high potential anion- or cation exchange capacity. They show an inhibition of organic matter mineralization as consequence of the low phosphorus availability, the strong association of soil organic matter with minerals (Matus et al., 2014), the low pH values and the resulting Al toxicity (Illmer et al., 2003; Tate, 1980). In addition, soil enzymes and microbial by-products may be deactivated by adsorption on short-range order (SRO) mineral surfaces (Saggar et al., 1994). Andisols are a specific kind of andic soils, as reported in USDA classification (Staff, 2008), that show unique physical, chemical and morphological properties due to their mineral phase. Soil minerals consist of short-range order materials such as allophane (with a nano ball structure), imogolite (with nano tube structure), ferrhydrite, oxides and oxyhydroxides of Fe and Al, and Al- and Fe-humus complexes, lacking long-range crystal atom order (Harsh et al., 2002). The mineralogical composition of the A horizons identifies allophanic and non-allophanic Andisols: the allophanic are dominated by allophane and imogolite-type materials, while non-allophanic by Fe- and Al organic complexes (humus complexes) (Matus et al., 2014). Allophanes are wethered metastable non crystalline materials that, after a weathering procedures, evolve to more stable crystalline minerals leading to other soil orders (e.g. Ultisols, Inceptisols, Alfisols) (Dahlgren et al., 2004).

The high SOM storage capacity of Andisols is function of the high surface areas of non-crystalline constituents that are available for the sorption of organic matter (Baldock & Nelson, 2000; Saggar et al., 1994). In Andisols the composition of

organic matter differs compared to other kinds of soils, in fact they accumulate more unsaturated C, more carboxyl and methoxyl functional groups from poorly degraded lignin, which may be involved in stable Al complex formation from amorphous materials (Conte et al., 2003). Many studies report that SOM in Andisols is composed by easily degradable microbial-derived material (Abelenda et al., 2011; Buurman et al., 2004; González-Pérez et al., 2007), as found also in non-volcanic soil. The alkyl structures are natural hydrophobic and, in Andisols, are protected by oxidation because are encapsulated into their hydrophobic network (Knicker & Hatcher, 2001). In allophanic Andisols, the stabilization of C and physicochemical protection of organic matter is due to minerals properties, especially SRO (allophane and imogolite type minerals) with a high reactive surface area (Parfitt et al., 2002; Torn et al., 1997; Zunino et al., 1982) joined to charged clay surface. The hypothetical model that explain Al-SOM complex formation in allophanic soils was explain by Matus et al. (2009): hydroxyl groups attracts or loose protons depending on the soil pH, therefore the reaction produces a negative charge that is compensated by electrostatic interactions with Na^+ or Ca^{2+} ions (Figure 5.1).

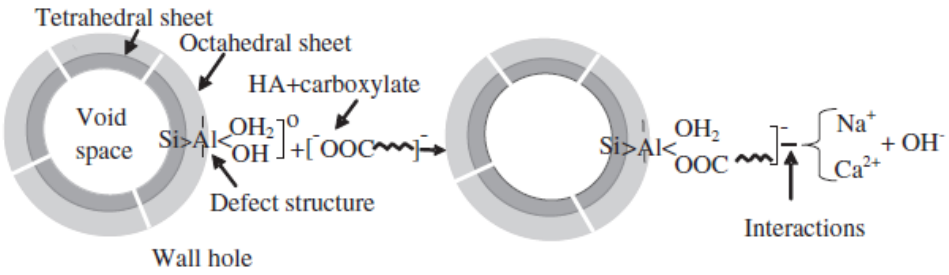


Figure 5.1 A transversal view of an external hypothetical allophane. The spherule and humic acids (HAs) with carboxylate groups (-COOH) show a ligand exchange mechanism and Na^+ or Ca^{2+} electrolytes by electrostatic interactions by (Matus et al., 2014).

The organic matter sorption is promoted by the presence of electrolytes such as NaCl and $CaCl_2$, so soil organic matter binds to the spherules of allophane and can interact again with a cation to form a new complex, mediated by electrostatic attraction. This

mechanism is the precursor of further electrostatic interactions for a new nucleus of Al-SOM complexes that subsequently precipitate. Moreover, also the Al oxides and climate factors are considered important stabilizing agents for SOM (Matus et al., 2008; Percival et al., 2000). Another mechanism involved in the SOC stabilization in volcanic soils is the electrostatic interaction with amorphous Al and clay minerals, as observed by Huygens et al. (2005) in Andisols from southern Chile, and is due to the simultaneous existence of positive and negative charges at field pH (Denef et al., 2002; Six et al., 2000b). The predominance of one of other mechanism is also related to the nature of Andisols (allophanic or non-allophanic soil).

The location of organic matter in stable aggregates is an important mechanism for SOM stabilization (Huygens et al., 2005). The aggregate hierarchy is considered a fundamental soil feature controlling various physical and biogeochemical processes, as proposed by Tisdall and Oades (1982) that hypothesized the formation of macro and micro aggregates in soils. According to this theory, microaggregates (20 to 250 μm) are composed by primary silt and clay particles that are bound together by persisting binding agents, like humified organic matter and polyvalent metal cation complexes; the bound of these particles by temporary and transient binding agents, such as fungal hyphae, roots, or microbial and plant derived polysaccharides, causes the formation of macroaggregates (>250 μm). The theory shows three main consequences: the first is the gradual breakdown of macroaggregates into microaggregates, if an increasing dispersing energy is applied to the soil; the second is the increase in percentage C concentration with increasing aggregates-size class, because large aggregates are composed by small aggregates size classes combined with organic binding agents; the third is that younger and more labile organic matter is contained in macroaggregates rather than in microaggregates (Elliott, 1986; Jastrow, 1996; Puget et al., 1995). This theory has been largely studied in soils dominated by crystalline minerals, like soil with a 2:1 dominant clay mineralogy, in which macroaggregates show younger and higher OC content than microaggregates

(exhibiting aggregate hierarchy utilizing organic matter as primary binding agent, (Six et al., 2002; Six et al., 2000a), but not in soils rich in poorly-crystalline or short range-order (SRO) minerals, like soils with dominant 1:1 clay mineralogy, exhibiting oxides as main binding agent, that do not show a higher organic C content in macroaggregates (Hoyos & Comerford, 2005; Six et al., 2000a). Therefore, this theory seems not applicable to Andisols, even if contrasting point of view could be found in literature. In fact, some authors report evidence of the absence of an aggregate hierarchy in studied Andisols, observing no differences in C concentration and $\delta^{13}C$ values among different size aggregates (Buurman et al., 2004); Huygens et al. (2005). focused attention on electrostatic attraction between and among Al and Al-oxides and clay minerals as main binding agents between soil aggregates in Andisols and not on the mineral humus complexes, because the aggregates formation is not related to total carbon content or allophane content, but to extractable-Al by oxalate, a procedure that allows the isolation only of amorphous and poorly crystalline Al-oxides and Al bound to organic complexes (Bertsch & Bloom, 1996). These results supported the idea that in Andisols SOC and metal humus complexes play a minor role in the aggregates build up (Denef et al., 2002), while there is a strong effect due to electrostatic attractions. As a consequence, the aggregate hierarchy is less pronounced. However in more recent study, Asano and Wagai (2014) have proposed a conceptual model for Andisols aggregate hierarchy. They affirm that in precedent studies the apparent lack of aggregate hierarchy is due to absence of variation in C concentration in different size aggregates after weak dispersion treatments (Candan & Broquen, 2009; Hoyos & Comerford, 2005; Huygens et al., 2005; Paul et al., 2008); they hypothesized that the aggregate hierarchy in Andisols is present at smaller spatial scales and maintained by stronger forces due to higher concentrations of SOM and nano-sized SRO minerals compared to non-volcanic soils. In fact, in their study, applying a stronger dispersion energy the macro- and micro-aggregates were breakdown liberating micron- to submicron-size particles, and the small particle (<53

μm) appeared enriched in N-rich organic matter, SRO minerals and organo-metallic complex. These results represent for authors an evidence of aggregate hierarchy in the studied soils. Anyway, the SOM physically protected is unaccessible for microbes, fungi and soil enzymes for the "tourtous" allophane structure in spite of the large pore volume and the large specific area (Chevallier et al., 2008). In Andisols the C storage and residence time change over time because mineral phase is subjected to a time weathering and aging processes that produces the formation of crystalline minerals. In general, during the early weathering stages, dominated by primary minerals, in soil occurs a maximum SOM accumulation (principally products of microbial metabolism); with the increase of poorly crystalline mineral, a sharply increase in SOM occurs (in this phase is higher the contribution of lignin in the organo-mineral complexes). Finally, the latest weathering stage is characterised by well crystallised mineral phase followed by a decrease in the SOM associated with soil minerals (Matus et al., 2014). A consequence of the natural evolution of the physico-chemical properties of Andisols is the variation over time of their C storage. Literature findings estimate the organic C turn over (170000 years) of allophanic soil larger 1000 time than the value measured for other kind of soils (Chevallier et al., 2008), as consequence of the unique properties and characteristics widely discussed above.

Another important characteristic of Andisols is the high P fixation and the low P availability; more than 50% of P incorporated in these soils is fixed as organic P (Borie & Rubio, 2003) and the P sorption capacity of an Andisol has been used as taxonomic criterion to define andic properties of soils (Takahashi & Dahlgren, 2016). Organic P (Po) in soil is found in the form of orthophosphate esters (C-O-P) including inositol phosphates, phospholipids, nucleic acids, phosphonates and organic polyphosphates. The turnover of organic P in soil is primarily determined by the rate of immobilization and mineralization (Condron et al., 2005). The P stabilization in soils is related to the sorption of P by ligand exchange of exposed Al-OH and -OH_2

groups (Beck et al., 1999), and it forms part of the humic substances by an interaction with OH and COO- groups to produce an inner sphere complexes and also by a direct interaction with mineral phase of the soil (Hamdan et al., 2012; Kirkby et al., 2011). In allophanic Andisols the sorption of P occurs on allophane or imogolite and Al-humus complexes (Takahashi and Dahlgren, 2016). Moreover the P sorption is pH dependent and archived the maximum around pH values between 3-4 (Nanzyo M., 1993). In general the mechanisms involved in organic P cycling in soil were separated from those for other organic matter components, included C. In fact, if organic N and S are directly bonded to C and can be released during microbial oxidation of organic C, organic P and some organic S are associated with soil organic matter in the form of ester (C-O-P) and are mineralizable only by specific enzyme, such as phosphatase (Condron et al., 2005).

Considering the above mentioned characteristics of Andisols, the aim of this research was to study the organic C stability and distribution in macro and microaggregates in three Chilean Andisols (Puerto Fonk, Pemehue and Piedras Negras), to evaluate how these soils respond to aggregate hierarchy, and to implement literature information about this topic.

5.2. Material and methods

5.2.1. Study area and soil sampling

The soil samples were collected from three Chilean Andisols (UFRO experimental site) in Southern Chile in La Araucania and Los Rios regions: Pemehue (PEH) (39°04` S and 072°10` W), Puerto Fonck (41° 28` S and 72° 59` O) and Piedras Negras (40° 19` S and 72° 56` O). In the past, all three sites were characterized by a forest land cover, today the actual land use are, for Puerto Fonck and Piedras Negras, permanent grassland (or graze), while for Pemehue annual cropping (wheat) under conventional tillage. In each site, three replicate samples were taken from the top layers (0-20 cm), air-dried and sieved at <2 mm mesh prior to analyses.

5.2.2 Dry sieved fractionation

All dry samples were sieved and separated into 4 size of aggregates: > 2 mm, 2 to 0.25 mm, 0.25 to 0.053 mm and < 0.053 mm. The sieving was performed by Sieve Shaker instrument (Retsch AS 200) equipped with a tower of three sieves placed, from the top to the bottom, in the followed order of mesh size: 2 mm, 0.250 mm and the finest of 0.053 mm. Each sample was sieved for 3 minutes at 150 rpm. This method allow us to separate the micro- (< 250 μm) and macro-aggregates (>250 μm), and in our case the size classes were 0.025-0.053 and <0.053 mm, >2 and 2-0.025 mm micro- and macroaggregates respectively.

After the sieving, all the separated fractions were weighed and it was calculated the % mass recovery:

$$\%\ Mass\ recovery = \frac{a_i}{X}\ x\ 100$$

Where a_i was the mass of a specific fraction and X was the total mass.

5.2.3. Hydrogen peroxide oxidation

The soil aggregates were oxidized by hydrogen peroxide. This oxidation is never complete and it has been used as the oxidation step in methods of mineralogical analyses (Kunze & Dixon, 1986). The organic matter resisting peroxide treatment consisted of organic interlayer complexes (Righi et al., 1995; Theng et al., 1992), so this treatment could be considered analogous to biological mineralization over the long term (Plante et al., 2004). In particular, 500 mg of > 2mm fraction and 200 mg of other three size dimensional fractions (2 -0.25 mm, 0.25-0.053, < 0.053 mm) were oxidized with 30% H_2O_2 adding in a flask respectively 37,5 ml and 15 ml and 75 ml and 30 ml of acid water solution at pH 2 (water acidified at pH 2 by HCl 0.1 M) with H_2O_2:suspension ratio of 1:2. The reaction was performed at the constant temperature of 60 °C for 16 h and on a magnetic stirrer. The mixing and the introduction in each flask of little glass bolls ensure homogeneous exposure of the organic matter to the oxidant and the breakdown of soil aggregates. At the end of reaction period, the

suspension was neutralized by NaOH 2 M and dialyzed against deionized water, to remove excess of H_2O_2 and salt. The dialysis was performed transferring quantitatively each sample in a dialysis bag of 1000 kDa membrane by a pipette and placing each one in deionized water until the achievement of constant value of water electrical conductivity. Each sample was later transferred into little plastic jars, frozen and then lyophilized. The dried samples were weighed and it was calculated the removed mass (%), with the purpose to evaluate the efficiency of OM oxidation for each fraction:

$$\%\, Removed\ mass\ = \frac{a_{i1} - a_{i2}}{a_{i1}}\ x\ 100$$

Where a_{i1} was the mass fraction before oxidation, and a_{i2} the mass fraction after oxidation.

5.2.4. Organic C and total N

The soil fractions were analyzed for total carbon and nitrogen content, using the flash combustion method performed by Elemental Analyzer EuroEA3000 series instrument. Prior the analyses, the samples were grounded in a agata mortar and sieving in a sieve of 160 µm mesh. About 2,5 mg and 1,5 mg respectively for < 2 mm and smaller fractions, were weighed in tin caps by analytical balance. To determine the C and N concentration in each sample, a calibration curve was performed using L-Cystine as standard and a soil certified reference material (C 4,40%, N 0,262%).

5.2.4. Total P content

The total phosphorus content was determined by hypobromite alkaline oxidation, using sodium hypobromite (NaBrO) (Dick & Tabatabai, 1977). Briefly, 100 mg of crushed sample were weighed in flask and added with 3 ml of NaBrO; the suspension was heated at 260-280°C (by hot-plate with sand bath) until the solution was totally dried, then from this time, they were continue heating for more 1 h. When the samples were cold, 4 mL of distillated water, 1 mL of formic acid (HCOOH) and 25

mL of 0,5 M H_2SO_4 were added. The samples were left overnight to the complete Br removal, and the next day, the total P was determined by the molybdenum blue method: 2,5 ml of each samples was added with 500 µl of NaOH 2N and 10 ml of molybdenum blue, the samples were left for 1 h until the spectrophotometric analyses by UV/visible spectrophotometer, and then the absorbance of each samples was measured at 750-800 nm (at typical molybdenum blue wavelength absorbance). The calibration standards were prepared at the same way.

5.2.4. Laser Scanning Confocal Microscope images

All the dry fractions, in one replicate for each experimental site, were observed at confocal microscope. The technique allows an image analyses in which the distribution of organic matter through soil fraction can be estimated thanks to the organic matter autofluorescence. The fluorescence is the property of some atoms and molecules to emit light at longer wavelengths after absorbing light of particular and shorter wavelengths (Herman, 1998). The absorption of a photon of energy, cause the excitation of the electron of a fluorescent molecule from the ground state to a higher electronic energy and vibrational state; the energized electron then returns to the ground energy state with a loss of vibrational energy to the environment and a photon of longer wavelength is emitted (Li et al., 2004). Some molecules are autofluorescent and emit fluorescence when excited (primary fluorescence, i.e. protein, nucleic acid, lipids). The image was taken by Laser Scanning Confocal Microscope (CLSN, fluorview 1000 CLSM), the light emitted by an excitation source is firstly absorbed by the sample and then emitted, in this way the autofluorescent OM is showed in light blue or green, and the color is related to different wavelength selected for the analyses and to chemical functional group in organic matter. Images were taken on one replicate for each Andisol.

5.2.5. Electrophoretic mobility

Electrophoretic mobility measurements were made using a Zetasizer Nano ZS apparatus (Malvern Instruments). The analyses was performed only for Piedra Negras site and for <2mm and <0.053 mm aggregates, before and after the H_2O_2 oxidation. Briefly, 1 mg of sample was suspended in 1 mL of 0.001 M NaCl by immersion in an ultrasonic bath for 5 min. Measurements were carried out over a range of pH values (between 3 and 10), adjusted by careful addition of either 0.01 M HCl or 0.01 M NaOH (Calabi-Floody et al., 2011)

5.2.6. Data analysis

Statistical analysis was performed using SigmaPlot 12.0 software. The differences among soil fraction and among experimental sites were tested by One way ANOVA followed by Student-Newman-Keuls test (significance level $P< 0.05$).

5.3. Results and discussions

The results of mass recovery for the four fractions in studied soils (Figure 5.2) showed that for all three sites the most abundant soil fraction (as percent of total dry weight) was 2-0.25 mm aggregate size ($P<0.05$), followed by 0.25-0.053 mm aggregate size. Moreover, the two soil under grassland (Puerto Fonck, *PF* and Piedras Negras, *PN*) showed an higher percentage of the fraction 2-0.25 mm compared to the cropland (Pemehue, PHE), on the contrary this latter site had a higher percentage of the fraction 0.25-0.053 mm ($P<0.001$).

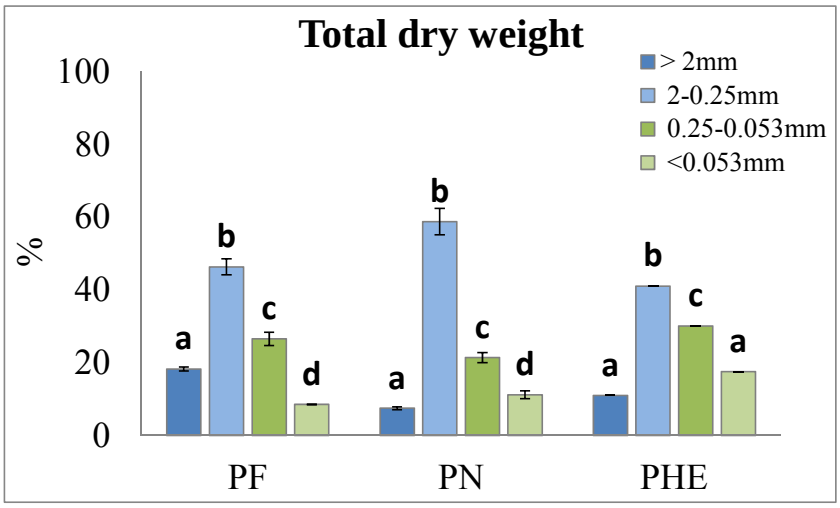


Figure 5.2 Mean values of mass recovery (± standard deviations) of the four soil fractions in the three studied Andisols: Puerto Fonck (PF), Piedras Negras (PN) and Pemehue (PHE).

The organic C (OC) content (Figure 5.3, A) showed no statistic differences among the soil fractions for both Andisols of Pemehue and Piedras Negras. On the contrary, in the Puerto Fonck Andisol (PF) the fraction with aggregate size 0.25-0.053 mm showed a significant ($P<0.01$) lower value of OC content compared to all other fractions, including the fraction with the smallest aggregate size (<0.053 mm), while the fraction with aggregate size >2 mm had the highest value of OC content.

Moreover, the four fractions with different aggregate size of PN and PHE Andisols were richer in OC content compared to those of PF Andisol (P<0,01).

No differences were observed in total N content (Figure 5.3, B) among the fractions of each soil and also comparing the three Andisols. In PF and PN Andisols, the C:N ratio (Figure 5.3, C) did not vary among the four fractions with different aggregate size, while the PHE Andisol showed a statistic reduction of C:N ratio in the fraction with the smallest size of aggregates (<0.053 mm, P<0.01) compared to the fraction with the biggest one (>2 mm). According with the results of organic C, in PF Andisol the fraction with aggregate size 0.25-0.053 mm exhibited the lowest value (P<0.01) of P content (Figure 5.3, D), whereas the fraction with the bigger aggregate size (>2 mm) showed the highest value (P<0.01) of P content compared to all other fractions. The PHE Andisol had the same behavior of PF, whereas in PN Andisol no difference among fractions were found. In PHE and PF Andisols, no difference in C:P ratio (Figure 5.3, E) was observed among the fractions, but higher values were generally found in PHE fractions (P<0.01) compared to PF.

With respect to the theory of aggregates hierarchy, Oades and Waters (1991) reported that soils responding to this theory exhibited a greater concentration of C and N in macro aggregates (>250 μm) than in micro aggregates and in bulk soil, as observed in Alfisol and Mollisol. The authors reported that this trend was more obvious with a more vigorous disaggregation treatment performed on their samples. If these observations are true for soils in which the organic materials are the dominant stabilizing agents in large aggregates (like Alfisol and Mollisol), in soils in which other stabilizing agents act (like oxides in Oxisol) they prevent the expression of aggregates hierarchy caused by organic materials (Oades & Waters, 1991). A similar behavior to Oxisol is expected in Andisol, because of in these soils Al- and Fe-oxides and SRO minerals are the main stabilizing agent for soil aggregates. In this study, we did not observe variations in OC and total N content among the four size classes of aggregates of PN and PHE soils, but PF soil showed a significantly higher OC

concentration in size fraction >2 mm compared to all other fractions, however the same result was not found for aggregate size 2-0.25 mm, that together the fraction with aggregate size >2 mm forms soil macro-aggregates (>250 µm).

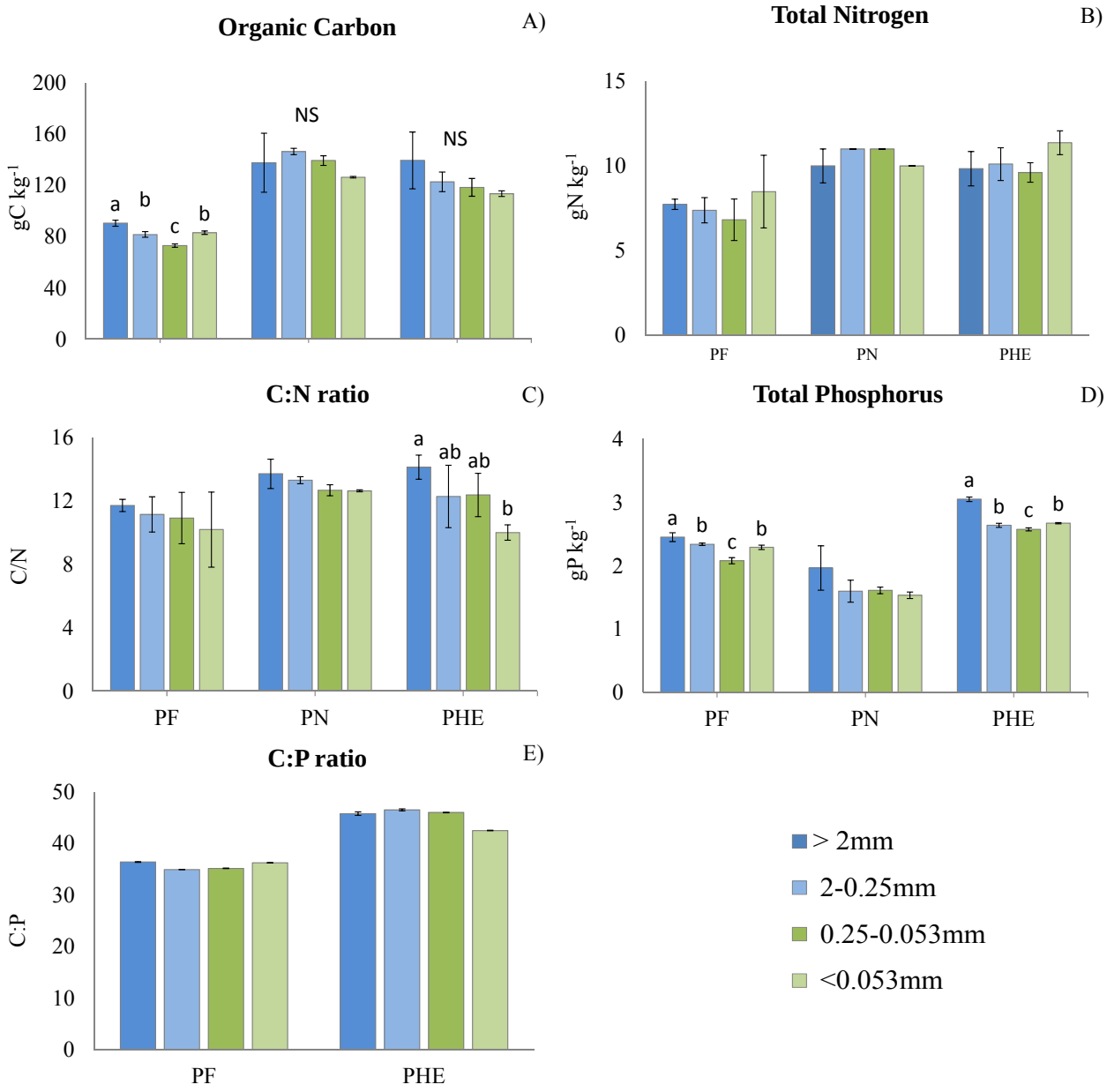


Figure 5.3 Mean values (± standard deviation) of the analysed chemical parameters: organic C content (A), total N content (B), C:N ratio (C), total P content (D), in the four size fractions of the three studied Andisols: Puerto Fonck (PF), Piedras Negras (PN) and Pemehue (PHE). C:P ratio (E) was calculated only for Puerto Fonck and Pemehue fractions. Different letters indicate significant differences (P< 0,01) among the fractions for each site.

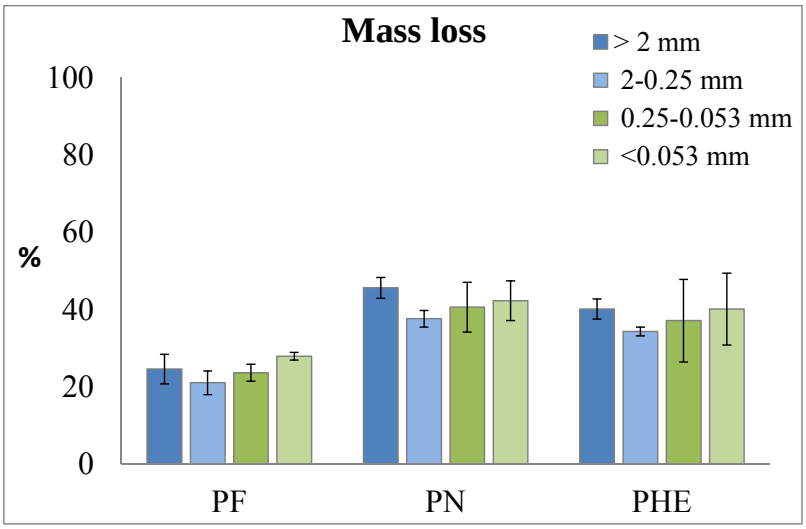
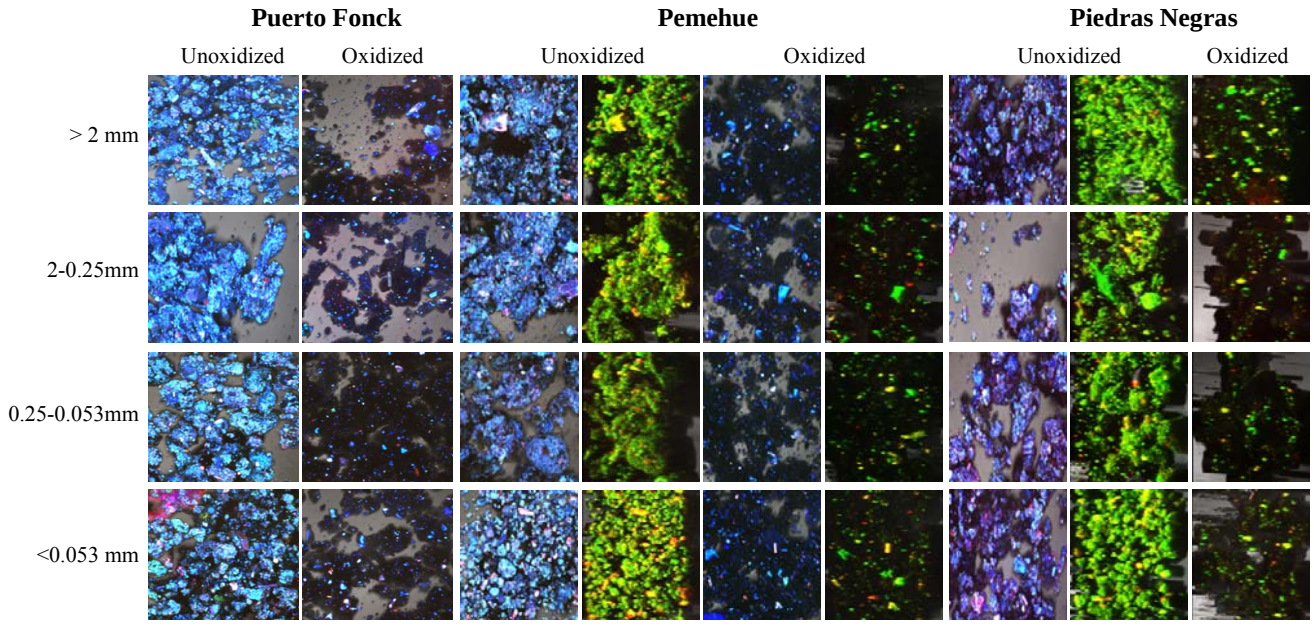


Figure 5.4. Mean values (± standard deviation) of mass loss, after H_2O_2 oxidation procedure, for the soil fractions of the three studied Andisols: Puerto Fonck (PF), Piedras Negras (PN) and Pemehue (PHE).

Figure 5.5. Laser Scanning Confocal Microscope images for the three Andisols, before and after the peroxide oxidation, foreach fraction with different aggregate size: row A) >2mm; row B) 2-0.25 mm; row C) 0.025-0.053 mm; D) < 0.053 mm. In the image the autofluorescence of soil organic matter was visualized in light blue or green (the different color is function of the chemistry and nature of the fluorescence molecule interested by the phenomenon).



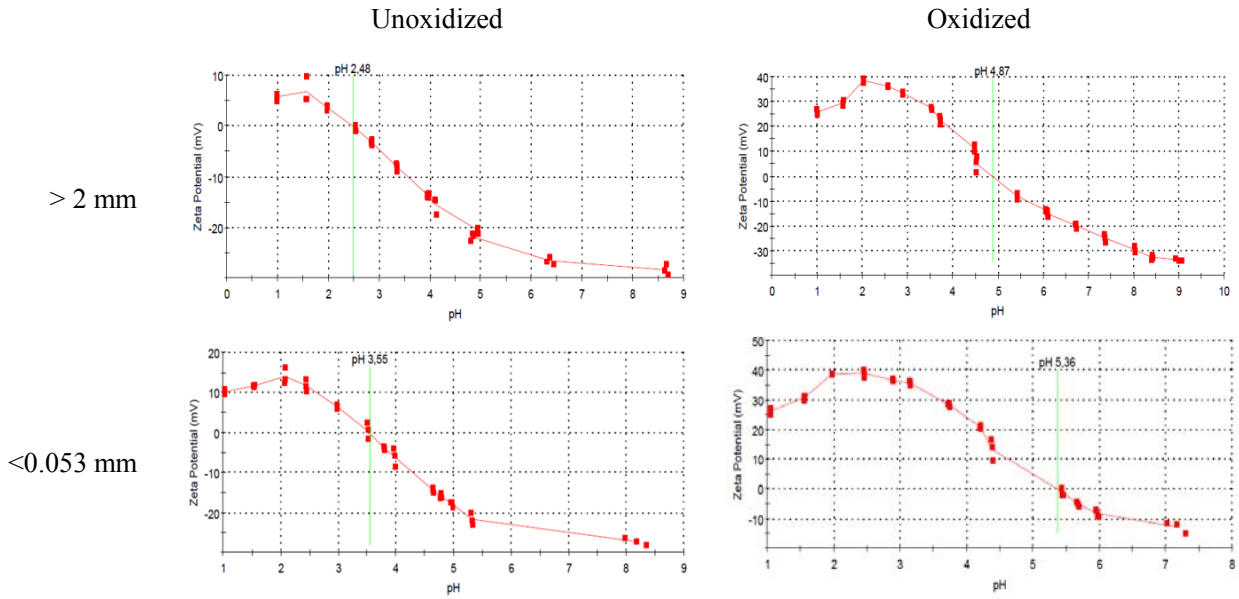


Figure 5.6 Zeta potentials as a function of suspension pH for >2 and >0.053 mm fraction of PF Andisol, before and after peroxide treatment. The green line indicate the isoelectric point

Our data are in agree with the study of Huygens et al. (2005) conducted on Chilean Andisols, that observed the highest mass recovery in the fraction with aggregate size 2-0.25 mm, and a total C concentration that was not significantly different among the fractions. Moreover they found that the macro aggregates contained only a small amount of the total OC content (2.4-8.3%), while the 56-74% of the total OC content of the soil was found in the remaining fraction, in addition also the $\delta^{13}C$ did not vary among the different size classes. A similar result was found by (Hoyos & Comerford, 2005) in Californian Andisols, where the authors observed that the studied soils did not follow the hierarchy of aggregates, but showed a variation in term of aggregate C content dependent from land use (with a pasture soil that showed more C incorporated into aggregates compare to a coffee-crop soil), even if the topography also affected the soil C storage. By contrast, our results did not show a clear differentiation between the behavior of cropland (PHE) and grassland (PF and PN) for all considered chemical parameters. The results of mass loss for the four fractions after the H_2O_2 oxidation (Figure 5.4) showed that in PN and PHE soils a higher percentage of organic matter was oxidized compare to PF soil, and that the

phenomenon was related to the higher OC content measured in PN and PHE soils (Figure 5.3, A) compared to PF. As concerns the different size fractions for each soil, an homogeneous removal of the organic matter occurred, indicating that the behavior of macro- and micro-aggregates in these soils, in term of organic C stability, was not affected by the aggregates dimensional size. A similar evaluation could be advanced observing the LSCM images taken on one replicate for each Andisols, before and after the oxidation (Figure 5.5). It was possible to observe in all aggregates of the three Andisols an homogeneous distribution of OM before the oxidation and an equal efficiency of OM removal after oxidation. These results corroborated the hypothesis that the OM stabilizing mechanisms act independently of aggregate size classes and that silt and clay size minerals are distributed evenly in all aggregates.

The zeta potentials for the finest (<0.053 mm) and the coarsest (>2 mm) fractions of PF Andisol before and after peroxide oxidation are shown in Figure 5.6. The value of zeta potential becomes more negative as the pH of the suspension increases, so it occurs an increase in the negative surface charge of allophane with a rise of pH (Calabi-Floody et al., 2011). The isoelectric point (IP) of the finest and coarsest fractions before the oxidation was respectively 3.55 and 2.48; after the oxidation the isoelectric point increase to 5.86 for the fine fraction and to 4.87 for the coarse fraction. The similar values of IP found in the two kinds of fractions, either before and after the oxidation, supports the observation that there was not a difference in organic matter content among aggregates, because a high amount of OM in a soil fraction generally determines a low IP, whereas the IP tends to increase if the proportion of minerals in soil is enhanced (Harbour et al., 2007; Jara et al., 2005; Theng et al., 2005). It was the phenomenon recorded after the samples oxidation, where increased in proportion the minerals because of the organic matter had been removed.

5.4 Conclusion

Our results confirm the not validity of aggregate hierarchy in Andisols and that differently from other kind of soil, in Andisols the separation of soil aggregates is very difficult. The general absence of changes in organic C, total N and total P contents, considering the macro- and microaggregates size classes, and the homogeneous distribution of OM before and after the oxidation, visualized by LSCM images, are all observations confirming the hypothesis that in Andisols Al- oxides and SRO minerals interactions strongly affect C stabilization and aggregates formation and that this stabilizing agents act in the same way in all aggregate size classes. Moreover in this specific soil, no differences were observed in soil under grassland or cropland, indicating that soil properties and soil specific characteristics in this environments affects more the organic C storage in aggregates size classes than the management.

References

Abelenda, M.S., Buurman, P., Camps Arbestain, M., Kaal, J., Martinez-Cortizas, A., Gartzia-Bengoetxea, N., Macías, F. 2011. Comparing NaOH-extractable organic matter of acid forest soils that differ in their pedogenic trends: a pyrolysis-GC/MS study. *European Journal of Soil Science*, **62**(6), 834-848.

Asano, M., Wagai, R. 2014. Evidence of aggregate hierarchy at micro-to submicron scales in an allophanic Andisol. *Geoderma*, **216**, 62-74.

Baldock, J.A., Nelson, P.N., 2000. Soil organic matter. In: Summer, M. (Ed), Hadbook of Soil Science. CRC Press Raton, FL, pp. B25-B84.

Beck, M., Robarge, W.P., Buol, S. 1999. Phosphorus retention and release of anions and organic carbon by two Andisols. *European Journal of Soil Science*, **50**(1), 157-164.

Bertsch, P.M., Bloom, P.R. 1996. Aluminum. in: *Methods of Soil Analysis Part 3—Chemical Methods*, (Eds.) D.L. Sparks, A.L. Page, P.A. Helmke, R.H. Loeppert, Soil Science Society of America, American Society of Agronomy. Madison, WI.

Borie, F., Rubio, R. 2003. Total and organic phosphorus in chilean volcanic soils fosforo total y fosforo organico en suelos volcánicos de chile. *Gayana Bot*, **60**(1), 69-78.

Buurman, P., Nierop, K., van Bergen, P., van Lagen, B. 2004. NaOH and Na4P2O7 extractable organic matter in two allophanic volcanic ash soils of the Azores Islands-a pyrolysis-GC/MS study. *O. Arnalds and H. Oskarsson*, 33.

Calabi-Floody, M., Bendall, J.S., Jara, A.A., Welland, M.E., Theng, B.K., Rumpel, C., de la Luz Mora, M. 2011. Nanoclays from an Andisol: extraction, properties and carbon stabilization. *Geoderma*, **161**(3), 159-167.

Candan, F., Broquen, P. 2009. Aggregate stability and related properties in NW Patagonian Andisols. *Geoderma*, **154**(1), 42-47.

Chevallier, T., Woignier, T., Toucet, J., Blanchart, E., Dieudonné, P. 2008. Fractal structure in natural gels: effect on carbon sequestration in volcanic soils. *Journal of Sol-Gel Science and Technology*, **48**(1-2), 231-238.

Condron, L.M., Tiessen, H., Turner, B., Frossard, E., Baldwin, D. 2005. Interactions of organic phosphorus in terrestrial ecosystems. *Organic phosphorus in the environment*, 295-307.

Conte, P., Spaccini, R., Chiarella, M., Piccolo, A. 2003. Chemical properties of humic substances in soils of an Italian volcanic system. *Geoderma*, **117**(3–4), 243-250.

Dahlgren, R.A., Saigusa, M., Ugolini, F.C. 2004. The Nature, Properties and Management of Volcanic Soils. in: *Advances in Agronomy*, Vol. Volume 82, Academic Press, pp. 113-182.

Denef, K., Six, J., Merckx, R., Paustian, K. 2002. Short-term effects of biological and physical forces on aggregate formation in soils with different clay mineralogy. *Plant and Soil*, **246**(2), 185-200.

Dick, W., Tabatabai, M. 1977. An alkaline oxidation method for determination of total phosphorus in soils. *Soil science society of America journal*, **41**(3), 511-514.

Elliott, E. 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. *Soil science society of America journal*, **50**(3), 627-633.

Eswaran, H., Van Den Berg, E., Reich, P. 1993. Organic carbon in soils of the world. *Soil science society of America journal*, **57**(1), 192-194.

González-Pérez, J.A., Arbelo, C.D., González-Vila, F.J., Rodríguez, A.R., Almendros, G., Armas, C.M., Polvillo, O. 2007. Molecular features of organic matter in diagnostic horizons from andosols as seen by analytical pyrolysis. *Journal of Analytical and Applied Pyrolysis*, **80**(2), 369-382.

Hamdan, R., El-Rifai, H.M., Cheesman, A.W., Turner, B.L., Reddy, K.R., Cooper, W.T. 2012. Linking phosphorus sequestration to carbon humification in wetland

soils by 31P and 13C NMR spectroscopy. *Environmental science & technology*, **46**(9), 4775-4782.

Harbour, P.J., Dixon, D.R., Scales, P.J. 2007. The role of natural organic matter in suspension stability: 1. Electrokinetic–rheology relationships. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **295**(1), 38-48.

Harsh, J.B., Chorover, J., Nizeyimana, E., 2002. Allophane and immogilite. *Dixon J.B., Schulze D.G. (Eds.), Soil mineralogy with environmental applications. Book Series No. 7. Soil Science Society of America, Madison*, 291-321.

Herman, B. 1998. Microscopy handbooks 40: fluorescence microscopy. BIOS, Oxford.

Hoyos, N., Comerford, N.B. 2005. Land use and landscape effects on aggregate stability and total carbon of Andisols from the Colombian Andes. *Geoderma*, **129**(3), 268-278.

Huygens, D., Boeckx, P., Van Cleemput, O., Oyarzun, C., Godoy, R. 2005. Aggregate and soil organic carbon dynamics in South Chilean Andisols. *Biogeosciences*, **2**(2), 159-174.

Illmer, P., Obertegger, U., Schinner, F. 2003. Microbiological properties in acidic forest soils with special consideration of KCl extractable Al. *Water, Air, and Soil Pollution*, **148**(1-4), 3-14.

Jara, A.A., Goldberg, S., Mora, M. 2005. Studies of the surface charge of amorphous aluminosilicates using surface complexation models. *Journal of colloid and interface science*, **292**(1), 160-170.

Jastrow, J. 1996. Soil aggregate formation and the accrual of particulate and mineral-associated organic matter. *Soil biology and biochemistry*, **28**(4), 665-676.

Kirkby, C., Kirkegaard, J., Richardson, A., Wade, L., Blanchard, C., Batten, G. 2011. Stable soil organic matter: a comparison of C: N: P: S ratios in Australian and other world soils. *Geoderma*, **163**(3), 197-208.

Knicker, H., Hatcher, P.G. 2001. Sequestration of organic nitrogen in the sapropel from Mangrove Lake, Bermuda. *Organic Geochemistry*, **32**(5), 733-744.

Kunze, G.W., Dixon, J.B. 1986. Pretreatment for Mineralogical Analysis. in: *Methods of Soil Analysis: Part 1—Physical and Mineralogical Methods*, (Ed.) A. Klute, Soil Science Society of America, American Society of Agronomy. Madison, WI.

Li, Y., Dick, W.A., Tuovinen, O.H. 2004. Fluorescence microscopy for visualization of soil microorganisms—a review. *Biology and fertility of soils*, **39**(5), 301-311.

Matus, F., Garrido, E., Sepúlveda, N., Cárcamo, I., Panichini, M., Zagal, E. 2008. Relationship between extractable Al and organic C in volcanic soils of Chile. *Geoderma*, **148**(2), 180-188.

Matus, F., Panichini, M., Godoy, R., Borie, F. 2009. Soil carbon storage in allophanic soils: study of a temperate pristine rainforest Nothofagus pumilio in the altitudinal limit. *Proceedings in ecological advances on Chilean temperate rainforests. Academic, Ghent*, 147-169.

Matus, F., Rumpel, C., Neculman, R., Panichini, M., Mora, M. 2014. Soil carbon storage and stabilisation in andic soils: A review. *Catena*, **120**, 102-110.

Nanzyo M., D.R., Shoji S. 1993. Chemical characteristics of volcanic ash soils. . *Volcanic Ash Soils - Genesis, Properties and Utilization Developments in Soil Science. Elsevier*, 145-187.

Oades, J., Waters, A. 1991. Aggregate hierarchy in soils. *Soil Research*, **29**(6), 815-828.

Parfitt, R., Parshotam, A., Salt, G. 2002. Carbon turnover in two soils with contrasting mineralogy under long-term maize and pasture. *Soil Research*, **40**(1), 127-136.

Paul, S., Martinson, G.O., Veldkamp, E., Flessa, H. 2008. Sample pretreatment affects the distribution of organic carbon in aggregates of tropical grassland soils. *Soil science society of America journal*, **72**(2), 500-506.

Percival, H.J., Parfitt, R.L., Scott, N.A. 2000. Factors Controlling Soil Carbon Levels in New Zealand Grasslands Is Clay Content Important? *Soil science society of America journal*, **64**(5), 1623-1630.

Plante, A.F., Chenu, C., Balabane, M., Mariotti, A., Righi, D. 2004. Peroxide oxidation of clay-associated organic matter in a cultivation chronosequence. *European Journal of Soil Science*, **55**(3), 471-478.

Puget, P., Chenu, C., Balesdent, J. 1995. Total and young organic matter distributions in aggregates of silty cultivated soils. *European Journal of Soil Science*, **46**(3), 449-459.

Righi, D., Dinel, H., Schulten, H.R., Schnitzer, M. 1995. Characterization of clay–organic-matter complexes resistant to oxidation by peroxide. *European Journal of Soil Science*, **46**(3), 423-429.

Saggar, S., Tate, K., Feltham, C., Childs, C., Parshotam, A. 1994. Carbon turnover in a range of allophanic soils amended with 14 C-labelled glucose. *Soil biology and biochemistry*, **26**(9), 1263-1271.

Six, J., Conant, R., Paul, E.A., Paustian, K. 2002. Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and Soil*, **241**(2), 155-176.

Six, J., Elliott, E., Paustian, K. 2000a. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. *Soil biology and biochemistry*, **32**(14), 2099-2103.

Six, J., Paustian, K., Elliott, E., Combrink, C. 2000b. Soil structure and organic matter I. Distribution of aggregate-size classes and aggregate-associated carbon. *Soil science society of America journal*, **64**(2), 681-689.

Staff, S.S.L. 2008. Soil survey laboratory methods manual. . *Soil survey Investigations Report. USDA SCS, Washington D.C., USA.*

Takahashi, T., Dahlgren, R.A. 2016. Nature, properties and function of aluminum–humus complexes in volcanic soils. *Geoderma*, **263**, 110-121.

Tate K.R., T.B.K.G. 1980. Organic matter and its interactions with inorganic soil constituents. *Soils with variable charge. Soil Bureau Department of Scientific and Industrial Research, New Zealand*, 225-244.

Theng, B., Yuan, G., Hashizume, H. 2005. Clay minerals and polymers: from soils to nanocomposites. *Clay science*, **12**(Supplement1), 69-73.

Theng, B.K., Tate, K.R., Becker-Heidmann, P. 1992. Towards establishing the age, location, and identity of the inert soil organic matter of a spodosol. *Zeitschrift für Pflanzenernährung und Bodenkunde*, **155**(3), 181-184.

Tisdall, J., Oades, J.M. 1982. Organic matter and water-stable aggregates in soils. *Journal of soil science*, **33**(2), 141-163.

Torn, M.S., Trumbore, S.E., Chadwick, O.A., Vitousek, P.M., Hendricks, D.M. 1997. Mineral control of soil organic carbon storage and turnover. *Nature*, **389**(6647), 170-173.

Zunino, H., Borie, F., Aguilera, S., Martin, J., Haider, K. 1982. Decomposition of 14C-labeled glucose, plant and microbial products and phenols in volcanic ash-derived soils of Chile. *Soil biology and biochemistry*, **14**(1), 37-43.

General conclusions

The preservation of the soil C stock is an important goal in future perspective in the framework of climate change, being the soil C sink the second C pool in terrestrial ecosystem after the oceans. The objective could be reach reducing the C losses or increase the C gains of a soil, and because of land use change and soil management practices are the human activity that most of all affect the soil C sink, the adoption of more conservative management strategy in agroecosystem could be a very important tool for its preservation. In particular this work focused on two aspects of this topic such as the potential in C sequestration of tree crop systems compared to crop land and the soil organic amendment, and both showed successful results. Olive groves was able to improve in long period (>30 years) the soil organic C stock in the upper horizons, and the soil organic matter fractionation, that allowed the separation of soil organic matter in three different pools, revealed a significant increase in the organic C stock not only in more labile fraction (as well as the light fraction), known to respond faster to land use change, but also in more stable forms (hydrolysate and recalcitrant fractions), that determine the duration for a long period of the C stock. The enrichment in radiocarbon content, that is the index of recently fixed C, in hydrolysate and especially in recalcitrant fractions compared to crop land and younger olive groves, indicated that really the olive grove improve the C sequestration in soil, even if the result could be influenced by the specific management strategy, preferring an intensive management to a super-intensively one. Also the employment of olive pomace, solid residue of olive oil extraction, appeared an effective agronomic practices to improve the organic C stock always in olive grove. Our study showed that after only 8 years of yearly amendment, the superficial C sink was significantly increased and also in this case allocated either in labile and more stable fractions. Moreover the pomace amendment positively affects soil microbial community in long period, essayed in term of microbial biomass, fungal biomass, microbial activity (such as rate of respiration and some enzyme activities)

without evident toxic effects in short period. However some negative effects also were observed as well as the activity related to N cycle (as revealed by the nitrification in potential conditions), the weak reduction in pH and the increase in electrical conductivity. The work underlined that despite some effects necessary to keep under control, the agronomic use of pomace could be a good strategy to restore the C sink in degraded agricultural ecosystem such as Mediterranean olive groves with the addition advantage to find an alternative use of olive by-product preventing its disposal. However the specific soil properties play an important role in soil C sequestration, as revealed by the different behavior along soil profile of one of the studied olive groves and by the particular case of Andisols. In Andisols the high potential in C sequestration is determined by the interaction and consequent stabilization of the organic matter on short-range order mineral and Al-oxides, much more abundant in this soil compared to other kind. As also focused by our work this property determine a difficult separation of soil in aggregates (as well as macro- and microaggregates) that respond to a specific behavior as reported in literature for aggregates from other kind of soil. In particular we observed an homogeneous organic matter distribution and organic C, total N and P concentration among the aggregates obtained in soil top horizons from all three Andisols studied, with also no great differences between land use (crop and pasture).

Therefore in order to ensure the achievement of preservation or improvement of soil C stock in agroecosystems by management practices, it is very important to consider soil specific condition and characteristics by preliminary study, because they constituent essential starting knowledges helpful to define and undertake the most safe and effective actions that allow to reach the prefixed objective.

Acknowledgements

This study and my doctorate was funded within the Italian national project "CARBOTREES PROJECT" (PRIN 2013-2016) and I want to thank all the members of the "unina2" research unit involved in the project and also the member of "unipg" unit. A special thanks goes to my supervisor Dott.ssa Rosaria D'Ascoli that gave me the chance to undertake this doctorate supporting my scientific activity and formation. I thank Dott. Fabio Marzaioli, the Institutes CIRCE (Centre for Isotopic Research on Cultural and nvironmental heritage) and the "Centro per lo Sviluppo ed il Trasferimento dell'Innovazione nel Settore dei Beni Culturali e Ambientali - INNOVA" that gave me the possibility to attend their laboratories to realize the Radiocarbon analyses.

I want to thank Prof. María de la Luz Mora for inviting me to her laboratory at the La Frontera University in Temuco (Chile) for the PhD stage.

The final thanks is for Prof. Maria Rao, Coordinator of Joint International Doctoral Program Italy-Chile in Environmental Resources Sciences for her efforts and devotion in this challenging project.