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MECHANISMS OF SOIL ORGANIC MATTER PROTECTION AND SEQUESTRATION

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DATA CONSEGNA TESI

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Riassunto

I meccanismi di protezione del carbonio organico del suolo rappresentano un argomento di grande attualità poiché, attraverso di essi, si esplica l'azione di *carbon sink* indicata nelle politiche volte alla mitigazione dell'effetto serra. Una maggior conoscenza di questi processi è quindi fondamentale per promuovere strategie di sequestro efficaci, così come previsto dall'articolo 3.4 del protocollo di Kyoto. Per realizzare lo studio è stato effettuato, a partire da macroaggregati prelevati da una prova di lungo periodo, un frazionamento degli aggregati stabili all'acqua con l'obiettivo di isolare dei *pool* di carbonio omogenei e riconducibili a specifici meccanismi di stabilizzazione. Sono stati frazionati in questo modo differenti suoli (argilloso, torboso e sabbioso) trattati con letame e con fertilizzanti minerali, ottenendo tre classi di aggregati (2000-250 μm , 250-53 μm and <53 μm). È stato quindi determinato, per i diversi aggregati, il carbonio organico (OC) e umico (HC), le frazioni umiche a diverso peso molecolare (HS), i gruppi funzionali presenti nelle sostanze umiche (spettroscopia NMR e DRIFT) e la porosità degli aggregati (porosimetria a intrusione di mercurio MIP). L'obiettivo è stato quello di valutare l'effetto delle differenti tesi sull'aggregazione e sulla distribuzione del carbonio organico, nonché valutare il ruolo delle sostanze umiche quali agenti leganti persistenti. Più in generale si è cercato di studiare quali meccanismi di protezione governano le diverse frazioni di aggregati. La tesi è stata strutturata in quattro capitoli; il primo capitolo è una *review* sul carbonio organico del suolo, i suoi meccanismi di protezione e le relative tecniche di analisi. Il secondo capitolo è dedicato alla distribuzione del carbonio organico e umico negli aggregati, delle frazioni umiche a diverso peso molecolare nonché del ruolo del carbonio umico come agente legante persistente. Il terzo capitolo è incentrato sull'analisi qualitativa degli estratti umici dei diversi aggregati sulla base dei gruppi funzionali rilevati dall'analisi NMR e DRIFT. Il quarto capitolo analizza le correlazioni fra la porosità dei diversi aggregati e le caratteristiche quali-quantitative del carbonio umico.

Summary

Soil organic carbon (SOC) protection processes have received much interest recently due to the possibility to enhance the carbon sink in agricultural soils with atmospheric CO₂ reduction purposes. Further knowledge of these mechanisms is thus fundamental to promote effective C sequestration practices in terrestrial sinks, as recognized by Article 3.4 of the Kyoto Protocol.

In a long-term experiment established in the early 1960s in north-eastern Italy, we used a combination of physical fractionation and chemical extraction techniques aiming to isolate homogeneous pools of SOC. In particular we wet-sieved large macroaggregates into three aggregate-size classes (2000-250 μm, 250-53 μm and <53 μm) in contrasting soils (clay, sandy and peaty) fertilized with manure or mineral fertilizers. We analyzed the organic (OC) and humic (HC) carbon of each aggregate fraction, the molecular weight of the humic substances (HS) extracted, the HS functional groups by NMR and DRIFT spectroscopy and the porosity of the different aggregate fraction by MIP (Mercury Intrusion Porosimetry). The aims were to evaluate the protection processes in the different SOC pools isolated analyzing the effect of the different fertilization types on the aggregates, the organic matter distribution and to investigate the composition and role of HC as binding agent. The thesis is structured in four chapters. The first chapter is a review on the SOC topic, its protection processes and the analytical methods for its study. The second chapter focuses on aggregate distribution, OC, HC, HS gel filtration and the role of HC as persisting binding agent. The third chapter had a qualitative description of C functional groups (NMR-DRIFT) in HS extracts from the different aggregate fractions. The final chapter had the correlation between the porosity of the different aggregate fractions and the different parameters measured in chapter II and III.

A Luisa con amore

Chapter I: Soil organic carbon protection processes and analysis techniques

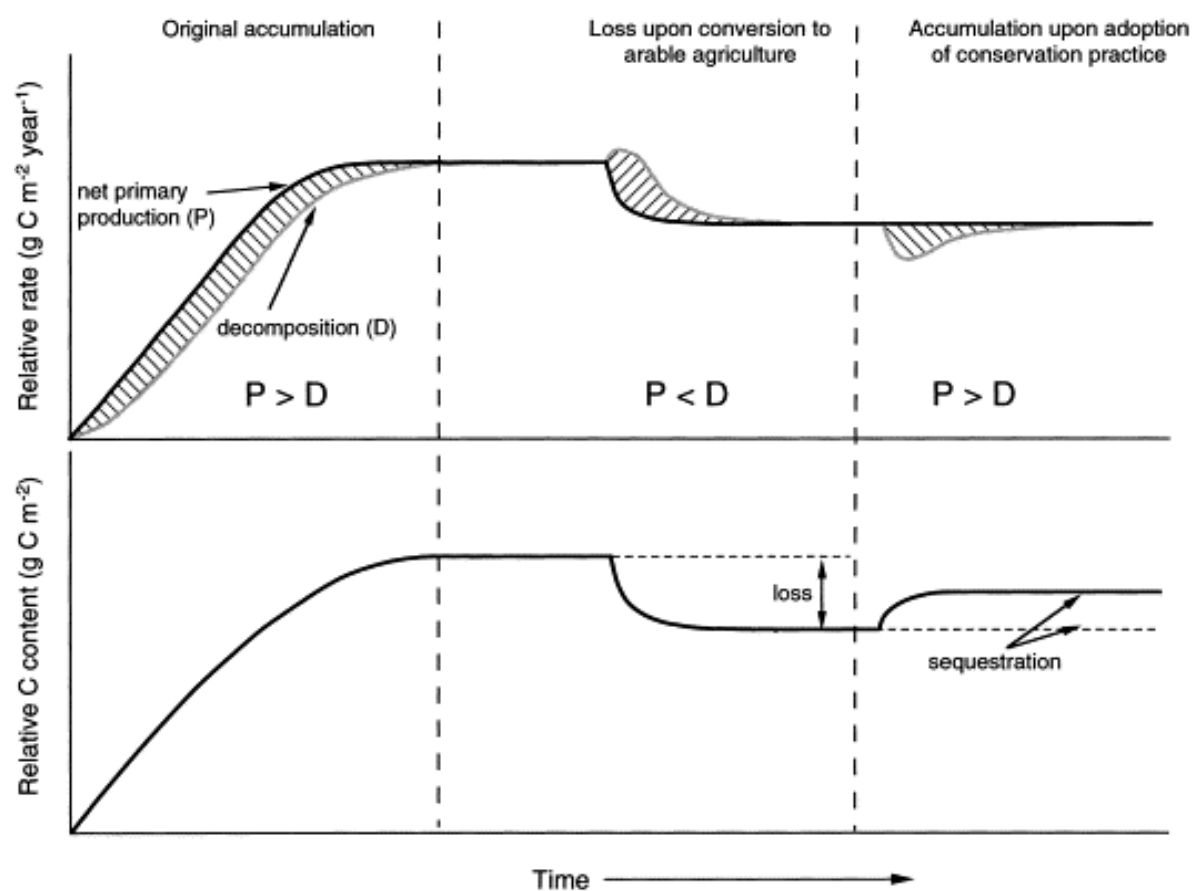
Introduction

The amount of soil organic carbon (SOC) stored in soils represents the third largest reservoirs of organic C on the global scale after oceans and the geological compartment (Schlesinger, 1995). The first meter of soil depth contribute with around 60% to the total carbon stored. This layer is directly influenced by agronomical activities. Field et al. (2007) estimated that the total global soil C pool including wetlands and permafrost (3,250 Pg C) is about four- to five-fold greater than the atmospheric (780 Pg C) and biotic (650 Pg C) pools. Consequently, any change in the size and the turnover rate of soil C pools may potentially alter the atmospheric CO₂ concentration and the global climate. Agricultural soils can act as a potential sink of the increased carbon dioxide in the atmosphere if managed properly by application of organic manures and balanced fertilizers. The Kyoto Protocol on climate change in 1992 (article 3.4) (Kyoto protocol, 1998) suggest to use the croplands as a carbon sink sites with appropriate managements systems which can decrease carbon dioxide evolution from soil to the atmosphere thereby lessening the impact on global warming and enhancing soil fertility and productivity (Lal 2004a). These policies can consider the use of zero or reduced tillage, improve efficiency of animal manure use and crop residue, application of compost to land, agricultural use of sewage sludge. In the article 3.4 there is a provision of assigning credits for C sequestration in agricultural soils (Oberthür and Ott, 2000). Thus, the C sequestered by soils can also be traded as a marketable commodity similar to other farm products. These C credits may be sold to comply with C offset protocols. In order to achieve that goal, establishment of a C databank for credible and verifiable rates of C sequestration in diverse land uses, different climates, and different soil management practices is needed along with identification of technological options that can enhance C sequestration in soils (Oberthür and Ott 2000).

SOC levels in soils reflect the long-term balance between additions and losses of organic carbon. Following the advent of traditional soil management, this long-term

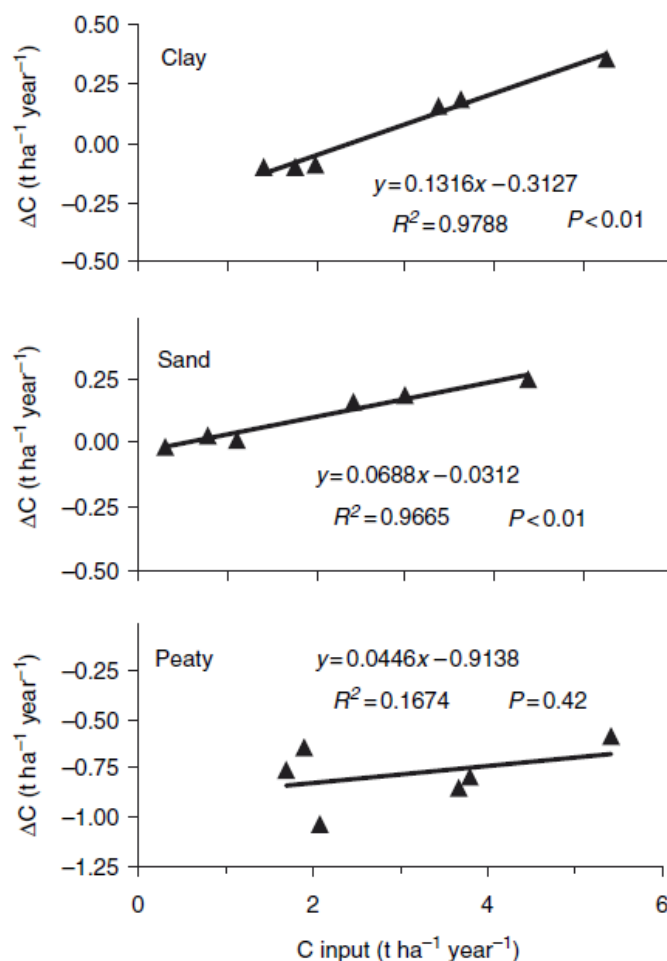
balance was disrupted and more and more of the C in the soil's organic matter was exposed to oxidative processes through continued cultivation. In this condition SOC stored was released into the atmosphere as CO₂. The SOC decreased until a new balance was approached (Figure 1) due to C input increase and/or modification in tillage practices.

Figure 1: SOC dynamics in a prairie agroecosystem. During soil formation, net primary production (P) exceeds decomposition (D), resulting in the accumulation of soil C until P and D again converge. Upon conversion of the land to arable agriculture, D initially exceeds P, resulting in the loss of soil carbon until a new steady state is approached. Adoption of C-retentive cropping practices reduces D relative to P, resulting in a gain of C until D and P again converge. (Janzen et al., 1997).



Many research have been conducted in the EU countries to evaluate the evolution of SOC in different soils and cultivation systems but few in Italy. Morari et al. (2006) analyzing data from two long-term trials observed a linear relationship between total C input and annual variation of SOC in clay and sandy soils (Figure 2). In the same trial an important decrease in SOC concentration after the introduction of intensive soil cultivation was observed, thus confirming a decrease in soil quality and the emission of CO₂ frequently reported for intensive arable farming. In the northern Italian plain the most promising RMPs are the conversion of arable land to permanent grassland and the regular use of organic manure.

Figure 2: linear relationship between total C in different soils



Triberti et al. (2008) evidenced that, in a long term trial, from 1972 to 2000 SOC stock did not change in the control and N fertilized plots, while it increased at mean rates of 0.16, 0.18, and 0.26 t ha⁻¹ year⁻¹ with the incorporation of residues, slurry and manure, corresponding to sequestration efficiencies of 3.7, 3.8 and 8.1% of added C with the various materials. They estimated that with the observed sequestration rate, manure application alone would take about 110 years to double SOC, unless a saturation condition is not reached in the meantime. Nevertheless the Authors reported that the large amounts of CO₂ released during manure maturation should be taken into consideration.

According with above observations, Bertora et al. (2008) found that the most C conservative management was the production and spreading of farmyard manure and that increasing the amounts of mineral N fertilizer did not affect the C sequestration rate. The authors measured the relationship between SOC and N stocks from 1999 to 2003 and the annual additions of C and N was described by the Hénin–Dupuis-based equation, evidencing that C and N retained in the soil each year varied by organic materials. The higher values were measured with farmyard manure (46% C and 44% N retained) .

All these agronomical techniques, recommended management practices included, need a deep knowledge of the mechanisms regulating the soil C stabilization to maximize the C sequestered into the soil. In 2012 the commitment period of the Kyoto Protocol will end and the international climate community has proposed various options to replace the Kyoto commitments. The new regime will need to encourage deep reductions in the release of greenhouse gases to prevent an increase in the Earth's average temperature that will result in serious negative impacts on ecosystems and human well-being. At the same time, it will need to ensure continued economic development in all countries and promote significant energy development in developing countries. This review aims to describe the evolution of SOC concept during the last centuries and to describe the mechanisms related to SOC stabilization and the state of the art in SOC pool isolation and analysis.

SOC concept during the centuries

The first occurrence of the term “soil organic matter” (SOM) in science cannot be dated with any precision. For example to Roman writers (Virgil, Pliny the Elder and Columella), “humus” meant “soil” or “earth”. Thus, Virgil named loamy soil “pinguis humus” and used the words “humus”, “solum” or “terra” interchangeably to convey the notion of soil or earth. 18 century later De Saussure (1804) ascribed a broad meaning to the word “humus” (the whole vegetative cover undergoing decomposition) and a narrow meaning to the word “mould” which referred to “the black substance plants are imbedded in”. Hundeshagen (1830) was the first to introduce a morphological classification of forest humus.

The changing meanings of humus reflect the evolution in the understanding and means of understanding of: (1) the nature and roles of SOM by scientists and to some extent of its management by agriculturists and (2) the changing human perception of, and relationship with, nature and the environment.

Until 1840, some still believed that plant dry matter was mainly derived from uptake of matter supplied by SOM, which was termed humus at that time. Agriculturists who believed this based the management of cropping systems fertility on the management of humus, i.e. through organic inputs. In 1809 Thaër proposed a “Humus Theory” that remained very influential for 30 years, as well as a quantified assessment of the agro-ecological and economic sustainability of farming systems. (2) From the 1840s to the 1940s, Liebig’s “mineral nutrition theory”, progressive abandonment of recycling of nutrients between cities and country, and breakthroughs in the processes of fertilizer industry paved the way for intensive mineral fertilization as a substitute for organic practices. The mineral fertilization theory was developed in a context of the demands of growing urban populations located in areas increasingly remote from those of plant production. Despite to this general tendency to discard the management of humus Schloesing in 1874 was probably the first that tried to isolate an organo-clay complex for the study of its properties observing that most of the total SOM was associated with clay and concluding that clay behavior depended on the quantity of OM associated with it.

The first concept related with SOM ability to improve bioavailability of mineral nutrients to plants was proposed by Grandeau (1878); this assertion left to SOM a new importance related with indirect effects on plant nutrition.

Although understanding of SOM and soil biological functioning was improving it had little impact on the rise of new mineral-based cropping patterns. (3) Since the 1940s, SOM has been gaining recognition as a complex bio-organo-mineral system, and as a pivotal indicator for soil and quality and agro-ecosystems fertility. Current characterization of SOM has largely moved away from operational definitions based solely on chemical extraction procedures, which give 'humic' and 'fulvic' acids. Instead, definitions based on physical fractionations are preferred as physical separation of SOM is related to the role that organic matter plays in soil structure and soil function. These fractionation procedures aim to partition SOM into components that differ in their longevity ('turnover time'), chemistry (structure and mass of molecules, types of functional groups), and origin (plant derived versus microbially derived). The study of the role of soils within the biogeochemical C cycle has made it obvious that we have an increasingly great need for better understanding and quantification of the reactions and mechanisms that control sources and sinks of nutrients, and of the biological, chemical and physical factors that regulate transformation processes, spatially and temporally, among these components.

Stabilization mechanisms of SOC

Stabilization is defined as protection of OM from mineralization; the amount of soil SOC is controlled primarily by two fundamental factors: input by net primary production (its quantity and quality) and its decomposition rate. The first decomposition have a turnover time of about 1–2 years in a temperate climate and it's accountable of the loose of 30-60 % the initial C, termed the active or labile OM pool (Jenkinson and Ladd, 1981). The second phase have a slow decomposition rates, with a total loss of about 90% OM and lasting about 10–100 years, is considered to be the intermediate OM pool. The complete decay process comprises a third phase and has a very slow decomposition rates and long

turnover times of about 100 to >1000 years. The SOC stabilization in soil depends on many mechanisms that are strongly interconnected. Abiotic chemical oxidation is likely to account for less than 5% of OM decomposition (Lavelle et al., 1993). Decomposition of natural OM in soils is mainly microbially mediated, with about 10–15% of the energy of organic C utilized by soil animals (Wolters, 2000). The chemical structure of organic molecules by itself is not sufficient to account for the extreme variation of soil OM in terms of age and turnover times.

Many authors aim to describe stabilization processes in separate classes. Most of them agree speaking about three major sets of processes simultaneously active: (1) selective preservation, (2) spatial inaccessibility and (3) interactions with surfaces and metal ions.

Selective preservation

(1) Selective preservation is the ability of some class of compounds to result recalcitrant to biotic and abiotic attacks. Plant litter and rhizodeposits, that can be considered interested by a primary selective preservation process, are composed of complex mixtures of organic components, mainly polysaccharides (starch, cellulose, hemicellulose and pectin; 50–60%) and lignin (15–20%), but also proteins, polyphenols (e.g. tannins), chlorophyll, cutin and suberin, lipids and waxes (10–20%) (Lützow et al., 2006). Some of these compounds are considered to be less biodegradable due to their structural composition. Molecular properties that influence decomposition rates of natural substrates are molecule size, polarity, ether-bridges, quaternary C atoms, three-fold substituted N-linkages, phenyl- and heterocyclic N-groups as well as long-chain (hydrophobic) hydrocarbons (Ottow, 1997). The polymers most resistant to degradation contain aromatic rings, such as in lignin and a range of polymethylenic molecules, such as lipids and waxes, cutin and suberin (Derenne and Largeau, 2001). Lignin contains no hydrolytic bonds but only aliphatic-, alcylyl- and biaryl-bonds that are accountable of a low decomposition rate by microbial enzymes. It can be accumulated during initial phases of residue decomposition (Melillo et al., 1989; Baldock et al., 1992). Therefore, Waksman (1938) and Umbreit (1962) concluded that stable humus compounds are formed predominately from persistent lignin components as well as

from fats and waxes from the original plant material. However, lignin concentrations in agricultural topsoils are very low, indicating that lignin is decomposed rapidly in these conditions (Kögel-Knabner, 2000). Recent studies using ¹³C CPMAS NMR and pyrolysis techniques have confirmed that lignin is altered relatively quickly. (Baldock and Nelson, 2000; Kögel-Knabner, 2000; Kiem and Kögel-Knabner, 2003).

Alkyl C, as for example in polymethylene structures, is considered a particularly recalcitrant form of soil C (Derenne and Largeau, 2001; Baldock et al., 2004).

The recalcitrance of alkyl C compounds is evident from the selective preservation of such compounds during biodegradation of soil OM, as reviewed by Baldock et al. (1997). This propriety is probably caused by the hydrophobicity of this compounds that can prevent access for degrading enzymes but also for the long chains that can be adsorbed on clay surfaces or be intercalated into phyllosilicates in a flat extended conformation. This effect is due to van der Waals interactions established between clay and polymer (Theng, 1979). It is not clear if the recalcitrance proprieties are conferred by hydrophobicity, or by intimate association with clay mineral surfaces. Probably both the mechanisms work together in the stabilization process. The accumulation of aliphatic materials in soils has been extensively demonstrated, and has often been associated with recalcitrance, but experimental evidence for the mechanisms responsible for this stability are lacking.

A secondary selective preservation could be associated to other carbon compounds classes like the (1) microbial biomass carbon (MC), (2) the humic substances (HS) and (3) the Charcoal (black carbon (BC)).

(1) MC represent between 0.3 and 7% of the organic C content (OC) (Wardle, 1992) and have a fast turnover time (<10 years) in the temperate zone (Jenkinson and Ladd, 1981; Coleman et al., 1983). For this reason the direct impact of microbial and faunal biomass on C stabilization can generally be regarded as minor (Scholes and Scholes, 1995; Wolters, 2000).

Despite this observation large part of the stable OM in soils is composed of microbially and faunally derived compounds. Microbial residues in soils contain

components specific for microorganisms, such as murein, chitin and lipids; these compounds have been shown to accumulate in soils (Kögel-Knabner, 1993; Guggenberger et al., 1994; Amelung et al., 1999; Marseille et al., 1999; Kiem and Kögel-Knabner, 2003). The larger part of the stable microbially derived OM in soils is composed by murein, chitin, lipids, melanins (produced by fungal activity) and algenans (aliphatic polyether components of algae). These compounds have similar stabilization mechanisms of their plant-derived analogues.

Different SOC pools are interested by humification process. They are transformed by chemical, biological, and physical processes into more stable compounds (Zech et al., 1997; Stevenson, 1994) normally indicated as (2) Humic Substances (HS).

(2) HS are considered to be refractory due to their chemical composition, thus belonging to the refractory SOM pool (Hayes and Clapp, 2001). They are synthesized by spontaneous heteropolycondensation processes catalysed by exoenzymes that produce chemical structures that are different from precursor plant polymers; these structures are not degradable with the normal microbial enzymatic 'toolbox' and are therefore recalcitrant (Hedges, 1988; Stevenson, 1994). New theories (Piccolo, 2002) about the supramolecular structure nature of HS consider this class of complexes as made by the self-assembling of heterogeneous and relatively small molecules. According to this conceptual model, small molecules deriving from the decomposition of plant and microbial residues form clusters by hydrophobic interactions and by hydrogen bonding resulting in the apparent large molecular size of humic substances ('pseudo-macromolecularity'). At present it is not clear how these pseudo-macromolecules would be stabilized in soils.

Charcoal (black carbon (BC)) represents a class of compounds involved in the SOC selective preservation. It is composed by a range of complex, highly condensed aromatic chemicals with a residence time estimated in the order of 500–10 000 years in soils. Their recalcitrance properties are probably due to their own chemical composition and the oxidized nature. Some authors (Hamer et al., 2004) showed a much greater rate of decomposition of BC related with the presence of easily available C source (glucose), thus show a recalcitrance induced

by the lack of energy substrates. The interaction with minerals could be responsible for the stabilization of partly degraded charred OM (Brodowski et al., 2005b).

The primary and secondary selective preservation of OM do not explain the long-term stabilization of potentially labile compounds observed by many authors. This conclusion is in contrast to Krull et al. (2003), who suggested selective preservation due to the recalcitrance of OM as the only mechanism by which soil organic C can be protected for long periods of time.

OM protected by spatial inaccessibility

Important processes that can reduce soil OM accessibility are (1) the occlusion of OM by aggregation, (2) the intercalation of OM within phyllosilicates, (3) the hydrophobicity of OM and (4) the encapsulation in organic macromolecules.

Aggregation (1) of soil particles and OM is very important because most temperate soils are aggregated and there is common evidence that soil structure protects OM from degradation; as consequence C mineralization is enhanced when soil aggregates are disrupted (Elliott, 1986; Gupta and Germida, 1988; Reicosky et al., 1997; Tebrügge and Düring, 1999; Six et al., 2000; Six et al., 2002b). Occluded OM into aggregates is spatially protected against decomposition due to: (1) reduced access for the microorganisms and their enzymes; (2) reduced diffusion of enzymes into the intra-aggregate space; and (3) restricted aerobic decomposition due to reduced diffusion of oxygen.

According to the hierarchical theory (Tisdall and Oades, 1982; Oades and Waters, 1991), stable microaggregates (<250 μm) are bound together to form macroaggregates (>250 μm) with organic compounds of different origin and stability. Microaggregates are assumed to be stabilized by persisting binding agents, whereas macroaggregates are stabilized by transient organic materials such as microbial- and plant-derived polysaccharides or temporary binding agents such as fungal hyphae and roots (Six et al., 2004). As a consequence, SOM concentration increases with increasing aggregates size, because macroaggregates contain microaggregates plus organic binding agents (Jastrow, 1996; Six et al., 2000).

Stabilization of OM within macroaggregates is very sensitive to management practices and restricted to soil horizons with continuous residue input. For these reasons agricultural activities could strongly affect the aggregate dynamic and OM balance. Turnover of OM in macroaggregates is much faster than in the microaggregates (e.g. Six et al., 1998; Six et al., 2002b; John et al., 2005) showing low or non OM physical protection processes. Recent studies indicate that the macroaggregate (>250 μm) structure exerts a minimal amount of physical protection (Beare et al., 1994; Elliott 1986; Pulleman and Marinissen, 2001), whereas SOM is protected from decomposition in free (i.e. not within macroaggregates) microaggregates (<250 μm) (Balesdent et al., 2000; Besnard et al., 1996; Skjemstad et al., 1996) and in microaggregates within macroaggregates (Denef et al., 2001; Six et al., 2000).

Microaggregates are considered very stable (Oades, 1993) due to their chemi-physical genesis and different physical properties; for this reason the lifetime of the pore system that traps the OM must also be considered long. For example small microaggregates are rich in pores <0.2 μm diameter, which is considered to be the limiting size for access by bacteria and their predators.

The intercalation of OM within phyllosilicates (2) is a process that involves 2:1 clays only at a pH <5. Organic ligands from enzymes, proteins, fatty acids or organic acids can be intercalated into the interlayer spaces of expandable phyllosilicates. The silt clay fraction is characterized by a high level of aliphatic compounds (C-alkyl); the main protection processes identified for this fraction are the interactions of OM with the mineral surfaces and the intercalation of OM into the interlayer spaces of expandable phyllosilicates. Chen et al. (2003) and Mahieu et al. (1999) reported that the abundance of alkyl C in soil organic matter increased systematically with decreasing particle size; Lützow et al. (2006) reports an estimated OM turnover in the silt clay fraction more than 100 years. The chemical characterization and quantification of OM after intercalation is highly unreliable (Leifeld and Kögel-Knabner, 2001) due to the difficulties in chemical characterization and quantification of this specific OM fraction. In general the future research must be

enhanced to explain this process that seem very important in SOC long term stabilization in acid soils.

The hydrophobicity (3) reduces surface wettability and thus the accessibility of OM for microorganisms. It's a propriety that is strongly related with OM chemical proprieties and especially specific compounds. For this reason it can be seen as a combined process of chemical selective preservation and physical spatial inaccessibility. Hydrophobic properties of soil OM have multiple effects on stabilization mechanisms, namely reduction of access by microorganisms, protection of labile molecules, and enhancement of aggregate stability. A similar physical protection of labile organic matter is made by their encapsulation in the network of recalcitrant polymers or humic pseudo-macromolecules (4) (Knicker et al., 1996; Zang et al., 2000). However, there is only limited evidence for the occurrence of encapsulation of labile OM in organic pseudo-macromolecules in soils and the verification of the process of protection difficult to demonstrate.

Stabilization of OM by interaction with mineral surfaces and metal ions

Various mechanisms are considered for interactions of OM with mineral surfaces, i.e. ligand exchange, polyvalent cation bridges, and weak interactions, such as hydrophobic interactions including van der Waals forces and H-bonding (Theng, 1979; Oades, 1989; Vermeer and Koopal, 1998; Vermeer et al., 1998). Soil OM in fine silt and clay fractions is older (Anderson and Paul, 1984; Scharpenseel and Becker-Heidmann, 1989; Quideau et al., 2001; Eusterhues et al., 2003) or has a longer turnover time (Balesdent et al., 1987; Balesdent, 1996; Ludwig et al., 2003) than OM in other soil OM fractions (Chenu and Stotzky, 2002). Chenu and Stotzky (2002) suggest that small molecules sorbed to mineral surfaces cannot be utilized by microorganisms unless they are desorbed so that they can be transported into the cell. The adsorption of macromolecules is considered non-reversible (Chenu and Stotzky, 2002) and associated with conformational changes that render macromolecules unavailable to the action of extracellular enzymes (Theng, 1979; Khanna et al., 1998).

Metal ions that have been considered as potentially stabilizing for soil OM are Ca²⁺, Al³⁺ and Fe³⁺ (Baldock and Skjemstad, 2000) and heavy metals. The effects of

metals on OM stabilization are still poorly understood and difficult to differentiate, with changes in substrate quality by complexation with metals, direct toxic effect of metals on soil microorganisms, and direct effects of metals on extracellular enzymes all described. Changes in molecular size, charge and steric properties of soil OM induced by metal complexation will probably decrease their accessibility to soil enzymes (McKeague et al., 1986) and thereby reduce their availability as microbial substrates. Evidence for soil OM stabilization by metal ions interactions is available, but general conclusions on their quantitative relevance in soils are difficult to draw.

The simultaneous action and interaction of the different stabilization processes is likely the major obstacle to elaborate fractionation schemes that could isolate functional and homogenous SOM pools (Lützow et al., 2007).

SOM physical fractionation methods

SOM consists of various functional pools that are stabilized by specific mechanisms and have certain turnover rates as seen before. Turnover rates (k) may be determined by different methods: (a) decomposition studies, (b) natural labelling of SOM using stable ^{13}C tracers, (c) in situ labelling of SOM with 'bomb' ^{14}C and (d) the ^{14}C -dating technique. Decomposition studies (a) of litter mostly quantify the short-term decomposition and consequently the turnover of the active pool, which is highly dependent on residue quality (Jenkinson, 1971; Ladd et al., 1983; Swift et al., 1979). Depending on the authors, the pools are termed as active, intermediate or slow and passive or inert (McGill, 1996; Smith et al., 1997).

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. Physical fractionation involves the application of various degrees of disaggregating treatments (dry and wet sieving, slaking), dispersion (ultrasonic vibration in water), density separation and sedimentation. We can divide them in (1) aggregate fractionation, (2) Particle

size fractionation, (3) Density fractionation and High-gradient magnetic separation (HGMS).

Aggregate fractionation

Aggregate fractionation (1) is based on the separation of free SOM and protected SOM that is occluded in secondary organo–mineral assemblages of different sizes. The different aggregate fractions aim to isolate active from intermediate and passive SOM pools and are obtained by dry or wet sieving and slaking. To identify SOM pools that preferentially stabilize SOM in the longer term, Six et al. (2000a) suggested a fractionation scheme of wet sieving and slaking to completely break up macroaggregates while minimizing the breakdown of the released microaggregates (53–250 μm). Various studies have shown that turnover times revealed by ^{13}C natural abundance were about 15–50 years for OM stored in macroaggregates (>250 μm) and 100–300 years for OM in microaggregates (<250 μm) (Angers and Giroux, 1996; Besnard et al., 1996; John et al., 2005; Monreal et al., 1997; Puget et al., 2000; Six et al., 2002; Yamashita et al., 2006). In most temperate surface soils, aggregates generally do not break down into primary particles upon slaking (immersion in water of dry aggregates) but rather into smaller stable units, indicating that the aggregates are arranged in a hierarchical fashion (Oades and Waters, 1991; Tisdall and Oades, 1982). In these soils, macroaggregates (>250 μm) often contain more OM than microaggregates (<250 μm), because the former include microaggregates plus OM serving as an intra-macroaggregate binding agent (Cambardella and Elliot, 1993; Jastrow et al., 1996; Puget et al., 1995; Six et al., 2000).

Six et al 2002 describe the Soc protected in: (1) physically stabilized through microaggregation (53–250 μm sized), (2) intimate associated with silt and clay particles (0–50 μm silt and clay particles) and unprotected SOM through macroaggregation (250 μm sized).

Particle size fractionation

Particle size fractionation is based on the concept that SOM associated with particles of different size and therefore also of different mineralogical composition differ in structure and function (Christensen, 1992). While quartz particles that dominate the sand fraction exhibit only weak bonding affinities to SOM, the clay-sized particles (e.g. sesquioxides, layer silicates) 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).

Particle size fractionation provides a rough differentiation between young (active) and older (intermediate and passive) SOM. Slower C turnover rates in clay fractions compared to the sand fraction were explained by a combined action of all three process groups of OM stabilization: the chemical change in OM quality, an increase in spatial inaccessibility (e.g. due to microaggregation) and the adsorption of OM on mineral surfaces (Collins et al., 1999; Hassink et al., 1993; Kalbitz et al., 2005; Kleber et al., 2004; Laird et al., 2001; Sørensen, 1981; van Veen et al., 1985; Wattel-Koekkoek et al., 2003)

Density fractionation

Density fractionation is applied to isolate SOM that is not firmly associated with soil minerals (light fraction) from organo–mineral complexes (heavy fraction). The intention of density fractionation is to achieve active, intermediate and passive OM pools. Density fractionation has historically relied on organic liquids (tetrabromoethane $C_2H_2Br_4$, 2.96 g cm⁻³; bromoform $CHBr_3$, 2.88 g cm⁻³; tetrachloromethane CCl_4 , 1.59 g cm⁻³), but aqueous solutions of inorganic salts (Mg_2SO_4 , $ZnBr_2$, sodium iodide (NaI), sodium polytungstate $Na_6(H_2W_{12}O_{40})$ (SPT)) have become increasingly popular because of the toxicity of halogenated hydrocarbons (Christensen, 1992). Like aggregate and particle size fractionation, the density fractionation makes only a rough differentiation of active and passive OM.

High-gradient magnetic separation

High-gradient magnetic separation (HGMS) is a technique used to isolate clay fractions with different contents and crystallinity of Fe oxides that can be separated according to their different magnetic susceptibilities at different field strengths (Hughes, 1982; Schulze and Dixon, 1979; Shang and Tiessen, 1998).

The isolation of SOM stabilized by Fe oxides is of special interest because Fe oxides can form strong bonds by ligand exchange and provide the largest surface area in acid soils (Kahle et al., 2003; Kaiser et al., 2002b; Kleber et al., 2004; Torn et al., 1997). Studies with HGMS have been conducted only in tropical soils up to now, but the method seems to be very useful for differentiating the SOM fraction stabilized by Fe oxides.

SOM Chemical Extraction procedures

Many Chemical extraction methods are used to subdivide SOM pools based on their chemical properties. We can identify four major extraction; (1) dissolved organic matter (DOM) extraction, (2) soil microbial biomass carbon (MBC) extraction, (3) humic substances extraction and (4) Organic solvents extraction.

(1) DOM is defined as SOM $<0.45 \mu\text{m}$ in solution. Different extractants are used to obtain DOM, ranging from cold water to aqueous solutions of different ionic strength to simulate the soil solution. DOM is obtained in situ by extraction of soil solution in lysimeters and suction cups in the field. DOM consists of SOM ranging from small-defined molecules to colloidal substances. Hexose-to-pentose ratios indicated predominantly microbial origin of carbohydrates in DOC obtained from O, A and B-horizons (Guggenberger et al., 1994; Haynes and Beare, 1997; Huang et al., 1998). Although DOM represents a labile substrate for microbial activity (Burford and Bremner, 1975), only about 10–40% of DOC has been observed to be readily degradable (Haynes, 2005; Kalbitz et al., 2003; Marschner and Kalbitz, 2003). While the importance of DOC for C turnover in soils is beyond question, it is also obvious that DOC is not a homogeneous fraction (Balesdent, 1996; Haynes, 2005; John et al., 2003; Kaiser and Ellerbrock, 2005; Kalbitz et al., 2003). This,

together with the small amount of DOC in relation to bulk soil OC, makes it difficult to use DOC as a functional soil C pool with a defined turnover time.

(2) MBC is considered to be the chief component of the active soil OM pool (Smith and Paul, 1990). Methods for estimating the pool size of MBC are the chloroform-fumigation methods (Jenkinson, 1976; Vance et al., 1987b). Generally, MBC represents between 0.3% and 7% of SOC (Wardle, 1992). In forest ecosystems the MBC content ranges from 0.5–0.9% in O-horizons to 1.6–3.6% in A-horizons. Higher MBC and SOC contents in clayey soils (Franzluebbers et al., 1999; Parton et al., 1987) were attributed to the protection of microorganisms against predation as well as to the protection of SOM against decomposition due to organo–mineral interactions in finer textured soils (Campbell et al., 1991; Juma, 1993; von Lützow et al., 2002).

MBC is directly correlated with aggregation processes and on the characteristics (composition, origin) of different functional OM fractions (Lundberg et al., 2001) and further analysis may help to quantify the impact the microbial biomass and of microbial metabolites on stabilization processes.

(3) Humic substances are extracted using NaOH and Na₄P₂O₇ solutions; this approach is frequently used because they generally extract large quantities of humic material in most soils (Stevenson, 1994) and these amounts are sensitive to soil type (Olk, 2006). Humic substances are stabilized by humification processes and are considered to be refractory, thus belonging to the passive SOM pool (Hayes and Clapp, 2001). Humic substances subdivision in fulvic acid, humic acid and humin fraction is based on their solubility properties; The fulvic acid fraction is soluble in alkali (e.g. 0.1M NaOH+0.1M Na₄P₂O₇) and soluble in acid (e.g. HCl). The humic acid fraction is soluble in alkali and insoluble in acid. The humin fraction is insoluble in alkali (Stevenson, 1994). During NaOH extraction, H⁺-bridges within SOM are replaced by Na⁺, causing SOM solubilization and also a rearrangement of organic associations (Piccolo, 2002). Polyvalent cation bridges between SOM and soil minerals are not affected by NaOH. They are only disrupted by extraction with Na₄P₂O₇ (Schnitzer, 1978). NaOH extracted up to 80% of SOM, while extraction with Na₄P₂O₇ was usually less efficient, with up to 30% of SOM

(Kononova, 1966; Stevenson, 1994). Extraction with 0.5M NaOH removed more C and N from coarser fractions than from fine fractions. For extraction of humic substances, most often a mixture of e.g. 0.1M NaOH+0.1M Na₄P₂O₇ was used. OM extracted with NaOH is not a homogeneous soil fraction, because the extraction procedure simultaneously affects organo–mineral and organo–organo interactions. Na⁺ ions also interfere with the flocculation of clays, causing disaggregation. Compared to NaOH extracts, sequential extraction with NaOH and Na₄P₂O₇ seems to additionally separate SOM stabilized by polyvalent cation bridges on mineral surfaces or in clay microstructures (<20 μm). Investigations with soils of different texture and mineralogy would be necessary to examine the influence of the extraction procedure on the amounts and belonging of SOM.

(4) Different SOM pools can be isolated using different organic solvents. Repeated extractions of OM with n-hexane can isolate alkanes and fatty acids (Schnitzer and Schuppli, 1989). Dichloromethane/methanol extraction method was proposed for soil lipid extraction (Naafs et al. 2004, Wiesenberg et al. 2004a). Using these techniques Mahieu et al., 1999 identified that Alkyl C, lipids and waxes represents about 25% of SOM in surface soils. Lipids amount to about 2–6% of SOM and are assumed to be very important for OM stabilization (Baldock et al., 2004; Derenne and Largeau, 2001; Zech et al., 1985). Chloroform was proposed for extracting fatty acids, long-chain alcohols, and wax esters (Schnitzer and Schuppli, 1989).

Conclusions

SOM is a complex system that play a key role in environmental ecosystem and in the carbon cycle balance. Research on SOM components, it's stabilization mechanisms and dynamics must be managed with a inter-disciplinary approach due to its complexity and vastness. In particular the research strategy for a better understanding of SOM stabilization processes must therefore combine research on the molecular composition of soil OM in specific soil fractions with the different methods that are now available to provide a measure for SOM stability, such as turnover times or the age of soil OM. Currently available SOM fractionation methods generally isolate more than one functional SOM pool, they can only be

helpful in certain soil horizons where SOM is stabilized by a limited number of well-defined key mechanisms and where the fractionation method will differentiate between these pools. One of the possible way to isolate homogeneous SOM pools could be the combination of physical and chemical fractionation. Even with numerous approaches to improve and combine fractionation methods, a major remaining problem is that most procedures are not specific enough with regard to stabilization mechanisms.

However, there are a range of promising new methods such as the destruction of the mineral phase with HF, the characterization of SOM associated with pedogenic oxides by HGMS that yield more homogeneous SOM fractions.

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Chapter II: Distribution of organic and humic carbon in wet-sieved aggregates of different soils

Introduction

In recent years many efforts have been made to identify soil organic matter (SOM) fractions that are directly dependent on specific stabilization mechanisms. Of these, selective preservation, spatial inaccessibility and interaction with surfaces and minerals (Christensen, 2001; Six et al., 2002; Lützow et al., 2006; Lugato et al., 2008) are commonly recognized as the most important for regulating SOM turnover. Their simultaneous action and interaction is probably the major obstacle to elaborate fractionation schemes that could isolate functional and homogenous SOM pools (Lützow et al., 2007). However aggregate fractionation has become a very common method of investigation, due to the close link between aggregate formation and SOM turnover (Six et al., 2004). According to the hierarchical theory (Tisdall and Oades, 1982; Oades and Waters, 1991), stable microaggregates (<250 μm) are bound together to form macroaggregates (>250 μm) with organic compounds of different origin and stability. Microaggregates are assumed to be stabilized by persisting binding agents, whereas macroaggregates are stabilized by transient organic materials such as microbial- and plant-derived polysaccharides, or temporary binding agents such as fungal hyphae and roots (Six et al., 2004). As a consequence, SOM concentration increases with increasing aggregate size, because macroaggregates contain microaggregates plus organic binding agents (Jastrow, 1996; Six et al., 2000). Yamashita et al. (2006), using aggregate fractionation and maize isotopic signature in a C3 soil, reported a higher soil organic carbon (SOC) concentration in macroaggregates than microaggregates; the former contained a higher concentration of young maize-derived carbon especially in the particulate organic matter, confirming the role of decomposing plant material as transient binding agent (Abiven et al., 2009).

Humic substances (HS) are considered to be recalcitrant (Hayes and Clapp, 2001), rich in functional groups interacting with mineral surfaces and, for this reason, probably act as persistent binding agents. Thus, according to the hierarchical

concept, microaggregates should contain a higher proportion of HC (i.e. higher HC/OC ratio) than the other aggregate fractions.

Humic substances are operationally defined by a standardized extraction procedure, but recent studies have shown that they are a heterogeneous pool of substances with very different turnover times (Lützow et al., 2007). Thermal analysis, solid state nuclear magnetic resonance and pyrolysis have highlighted a heterogeneity of HC composition in the silt and clay fractions of the same soil and also among different soils (Mao et al., 2007). Kelleher and Simpson (2006), using advanced nuclear magnetic resonance approaches, suggested that the vast majority of operationally defined humic material is a very complex mixture of microbial and plant biopolymers and their degradation products, but not a distinct chemical category. Although knowledge on the interaction between SOC and the aggregation process is rapidly increasing, the role of humic substances as binding agents, probably because of their heterogeneity and controversial origin, has been less studied and is still unclear.

In a long-term experiment established in the early 1960s in north-eastern Italy, we wet-sieved large macroaggregates of three contrasting soils, fertilized with manure or mineral fertilizers, into three aggregate-size classes (2000-250 μm , 250-53 μm and <53 μm). We analyzed the organic (OC) and humic (HC) carbon of each aggregate fraction, also investigating the molecular weight of the humic substances HS extracted. The aims were to evaluate the effect of different soils and fertilization on: 1) soil aggregate distribution 2) organic matter distribution in the different aggregates 3); the role of humic carbon as binding agent.

Materials and methods

2.1 The long-term experiment

The long-term trial is located at the Experimental Farm of the University of Padova (Veneto Region, NE Italy). The local climate is sub-humid, with annual rainfall of about 850 mm and yearly average temperature of 12 °C. The reference evapotranspiration (ET_o) is 945 mm with a peak in July (5 mm d⁻¹). ET_o exceeds

rainfall from April to September. The site has a shallow water table ranging from about 0.5-1.5 m in late winter-early spring to 1-2 m in summer.

This experiment began in 1964 in 4 m² open lysimeters, 80 cm deep. The experimental treatments derive from the factorial combination of three types of soil (hereinafter called clay, sandy and peaty in relation to their dominant property in Table 1) with six types of mineral, organic or mixed fertilization, organized in two randomized blocks (36 lysimeters).

The soils were brought from three locations in the Veneto region: clay soil from the south-western plain, sandy soil from the central coastal area and peaty soil from the southern plain. The original soil profiles were reconstructed in the lysimeters. The sandy soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) contain predominantly quartz and feldspar and a significant amount of dolomite (16%). Clay soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) have a higher amount of montmorillonite (16%) than the other soils and a considerable presence of mica (19%) and dolomite (15%). The peaty soils (Typic Sulphisaprists euic, mesic – ARPAV, 2005) have a higher mica content (25%) and 12% of montmorillonite. After 40 years of experimental conditions, some of the original properties have changed, as reported recently in Morari et al. (2008) and in table 1.

Fertilization treatments considered for this study were as follows: no applications (O); farmyard manure – F2 (40 t ha⁻¹ yr⁻¹); mineral fertilizer – M2 (200 kg ha⁻¹ yr⁻¹ N - 100 P₂O₅ - 240 K₂O). The F2 applied about the same amount of macroelements as M2 and around 3.5-4 t C ha⁻¹ y⁻¹. Until 1984 there was a two-year maize (*Zea mays* L.) - wheat rotation (*Triticum aestivum* L.). Thereafter, a variable rotation was adopted between 1985 and 1992, with various horticultural crops. From 1993 to 2002 there was a three-year rotation of tomato (*Lycopersicon esculentum* Mill.) – sugarbeet (*Beta vulgaris* L.) – maize, followed by various horticultural crops, maize and sunflower (*Helianthus annuus* L.) from 2003 to 2007. Apart from fertilization, all plots were treated in the same way in terms of rotation and management (tillage, sowing, harvest, etc). The top 15-20 cm was dug each autumn and crop residues were removed from all treatments.

Table 1 Physical-chemical characteristics of the 0-30 cm depth at the beginning of the experiment (1964).

Parameters	clay	sandy	peaty
Sand (2 mm –50 μm) (%)	25.0	93.4	38.0
Silt (50-2 μm) (%)	23.0	6.0	13.6
Clay (<2 μm) (%)	52.0	0.6	48.4
pH	7.9 (7.7)	8.1 (7.7)	4.9 (7.2)
Total Carbonate (g kg^{-1})	26.0	39.0	0.0
Organic Carbon (g kg^{-1})	14.5 (15.7)	1.7 (3.1)	105.0 (89)
Total Nitrogen (g kg^{-1})	1.5 (1.5)	0.15	6.7 (6.5)
C/N	10.0 (10.2)	12.0	16.0 (13.6)
Total Phosphorus (g kg^{-1})	2.8 (5.6)	0.5 (1.8)	1.1 (3.1)
Available Phosphorus (mg kg^{-1})	161.5	26.2	100.4

Values between brackets were measured in 2002

2.2 Soil sampling and fractionation technique

The top soil layer (0-20 cm) was sampled in June 2008. The samples were taken from five different points in the lysimeter and bulked to obtain a sample of about 1 kg. Samples were broken up manually, air-dried and sieved at 2 mm. To standardize the fractionation procedure and better evidence the relationship between aggregation and organic binding agent in the different soils, we first isolated the large macroaggregates (from 1 to 2 mm) by dry-sieving. No large aggregates were isolated from sandy soil, so all the soil was retained. Large aggregates (two sub-samples per plot - 36 in total) were then separated into three aggregate size fractions by wet-sieving, using an automatic machine oscillator. Briefly, the apparatus produces vertical actions which transmit a 3 cm stroke vertical movement to a stack of interfitting sieves with 250 and 53 μm mesh. The

sieves, 10 cm in diameter and 5 cm height, were immersed in distilled water inside a beaker (15 cm diameter x 18 cm height). Prior to sieving, the water level was adjusted to prevent water reflux over the edge of the 250 μm sieve during the oscillation.

Before sieving, 15 g of sample were immersed on top of the 250 μm sieve for 10 min to allow slaking. After slaking, aggregates were separated by vertical oscillations at a frequency of 50 rpm for 18 min (for a total of 900 oscillations). Macroaggregates (2000-250 μm) and microaggregates (53-250 μm) were then collected from the corresponding sieves, whereas the silt-clay fraction (<53 μm) remaining in the beaker was precipitated by adding 3 ml of 1N CaCl_2 . All isolated fractions were dried at 60 $^\circ\text{C}$ in a forced-air oven. As the sand contents differed among aggregate-size classes, SOC concentrations were corrected to minimize the confounding effects of sand dilution:

$$\text{sandfree } C_{\text{fraction}} = \frac{C_{\text{fraction}}}{1 - \text{sand proportion}_{\text{fraction}}}$$

Sand correction was also tried for the sandy soil, but corrected SOC values were abnormally high and erratic. The denominator (*1-sand proportion*) was close to 0 because of the very high sand content (about 96%), so C content was not corrected for sand in this soil, in which the <53 μm fraction was also not recovered due to its very low amount.

For the clay and peaty soil, the water stable aggregates index (WSA) was calculated according to Kemper (1966):

$$WSA = \frac{M_{\text{weight}} - \text{sand}}{\text{soil}_{\text{weight}} - \text{sand}} \cdot 100$$

where M is the dry weight of 2000-250 μm aggregates.

2.3 Soil chemical analysis

Aggregates were analyzed for SOC (g SOC/kg dry soil) by dichromate oxidation (Walkley and Black, 1934). The humic substances were extracted from the air-dried samples with 0.5 M NaOH (1:10 w/v) in a Dubnoff bath at 50 $^\circ\text{C}$ for 16 h and separated from the suspended material by centrifuging at 15000 rpm for 15 min.

Here, the term humic substances is applied to the fraction soluble alkalis and comprehensive of humic and fulvic acids. Humic extracts (50 ml) were transferred into 18,000 mol. wt cut-off dialysis Visking tubing (Medicell Ltd., London, UK) and dialyzed against double-distilled water. The water was changed daily until the liquid outside the dialysis tube was colourless. the retained solution was then desalted by ion exchange on Amberlite IR 120 H⁺ (Stevenson, 1994).

Gel-permeation chromatography of humic extract was conducted on a Sephadex G-100 gel packed in a 70 x 1.6 cm Pharmacia column (Pharmacia, Uppsala, Sweden). The gel packing solution and eluent were 0.02 M Na₂B₄O₇. The apparent molecular weight of the fractions was: above 60 kDa (HF1); between 60 and 30 kDa (HF2) and less than 30 kDa (HF3). Column calibration was based on previously assessed standard proteins (Kit MS-II, Serva, Heidelberg, Germany) (Martin et al., 2006). Humic fraction analysis was done for each soil fraction, except for the not recovered <53 µm one of sandy soil.

2.3 Statistical analysis

Soil data were analyzed with two-way ANOVA considering: 1) 'aggregate class' x 'fertilization treatment' as factors and aggregate weight, OC, HC and OC/HC as variables; 2) 'HS molecular weight' x 'fertilization treatment' as factors and % of HS in macro, micro and <53 µm fraction as variables. Significantly different means were differentiated with the Student-Newman Keuls test (StatSoft Inc., 2004). The three soils were analyzed separately. To homogenize variances, percentage repartition of humic molecular weight fractions was subjected to angular transformation prior to ANOVA.

3. Results

3.1 Aggregates and organic-humic carbon distribution

In the clay soil (Fig. 1a) macroaggregates were clearly the dominant fraction (P<0.05), with a significant interaction fertilization x aggregate class. The manure application increased the macroaggregate proportion compared to the mineral

treatment, whereas it did not affect the <53 μm size fraction. In the O and M2 treatments there was a redistribution towards microaggregates, which was significantly different from the manured one. In the peaty soil (Fig. 1b), the macroaggregate fraction was also dominant ($P < 0.05$), with an average value of 432 g aggr. kg^{-1} of soil, however lower than that measured in the clay soil (574 g aggr. kg^{-1} of soil). The significant interaction showed the lower value of <53 μm fraction in the F2 treatments with respect to M2. In the sandy soil, the separated fractions, in practice representing particles size and not aggregates, were homogenous with respect to fertilization (Fig. 1c).

In both clay and peaty soils (Fig. 1a, Fig. 1b), the <53 μm fraction was significantly depleted in SOC with respect to the micro and macroaggregates ($P < 0.05$). These two aggregate classes showed comparable values between treatments, except for the manured one in the clay soil where the highest values were measured in the macro and microaggregates (21.75 and 27.02 g C kg^{-1} aggr. respectively). A similar trend was observed for the peaty soil (Fig. 1b). Larger (2000-250 μm) and intermediate (53-250 μm) fractions of sandy soil showed no significant statistical difference within fertilizations on SOC concentration; however the manure application resulted in a higher average SOC concentration than the O and M2 treatments ($P < 0.05$).

In all soils, HC was closely correlated ($r = 0.98$) to OC considering the different aggregate fractions and fertilizations (Fig. 1a-b-c). As a consequence the HC/OC ratio revealed no clear pattern in relation to aggregation, since the factor 'aggregate class' was not significant in either clay or peaty soils. However higher values (significant interaction fertilization x aggregate class) were observed in the macroaggregates of F2 and M2 in the clay and peaty soils, respectively. The values in these soils ranged narrowly between 0.3 and 0.6, whereas very homogenous values were measured in the sandy soil. The fertilization factor was significant only in the clay soil, with an average ratio of 0.49 and 0.44 in the F2 and M2 treatments, respectively, significantly higher than in the O treatments (0.37).

Figure 1a –Clay soil; Water-stable aggregate distribution (g aggregates kg⁻¹ sand-free soil), CO and HC aggregate concentration (g C kg⁻¹ sand-free aggregate) and HC/CO ratio in the different soils and treatments; macroaggregates 2000-250 μm, microaggregates 250-53 μm, silt-clay fraction <53 μm. Histograms with the same letters, referring to ANOVA interaction ‘aggregate class’ x ‘fertilization treatment’, are not statistically different at P<0.05. Bars represent the standard error.

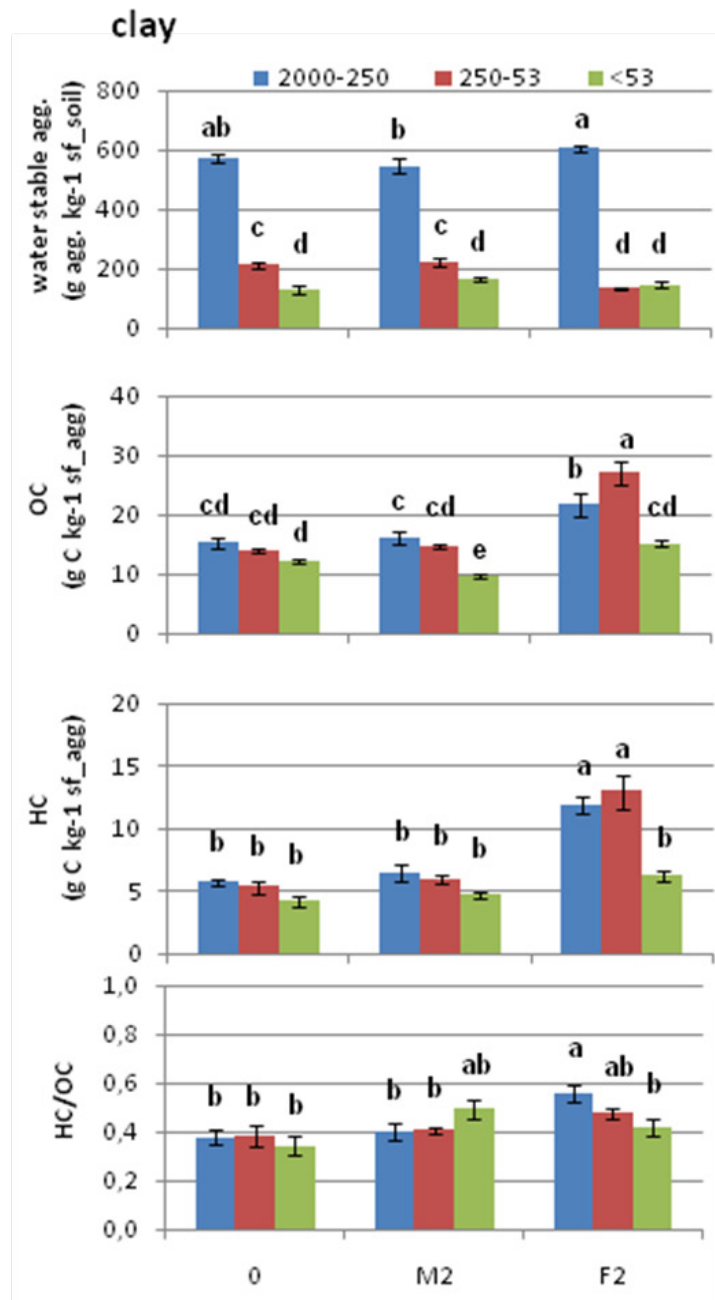


Figure 1b –Peaty soil; Water-stable aggregate distribution (g aggregates kg⁻¹ sand-free soil), CO and HC aggregate concentration (g C kg⁻¹ sand-free aggregate) and HC/CO ratio in the different soils and treatments; macroaggregates 2000-250 μm, microaggregates 250-53 μm, silt-clay fraction <53 μm. Histograms with the same letters, referring to ANOVA interaction ‘aggregate class’ x ‘fertilization treatment’, are not statistically different at P<0.05. Bars represent the standard error.

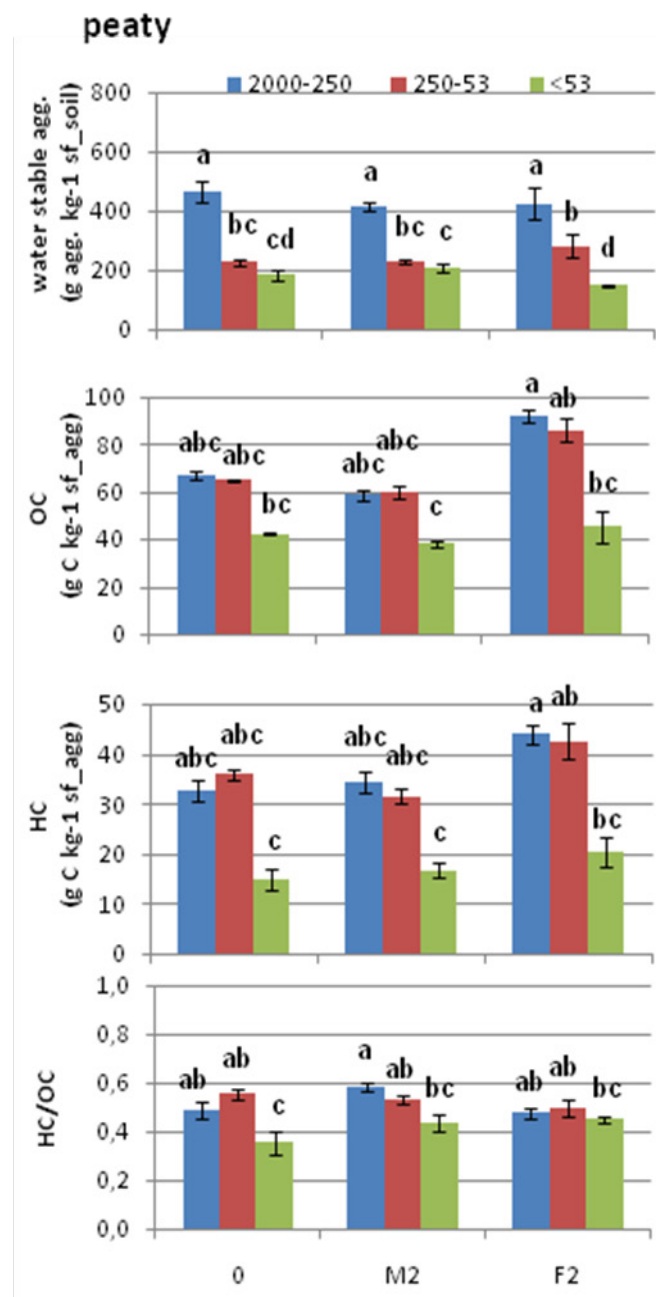
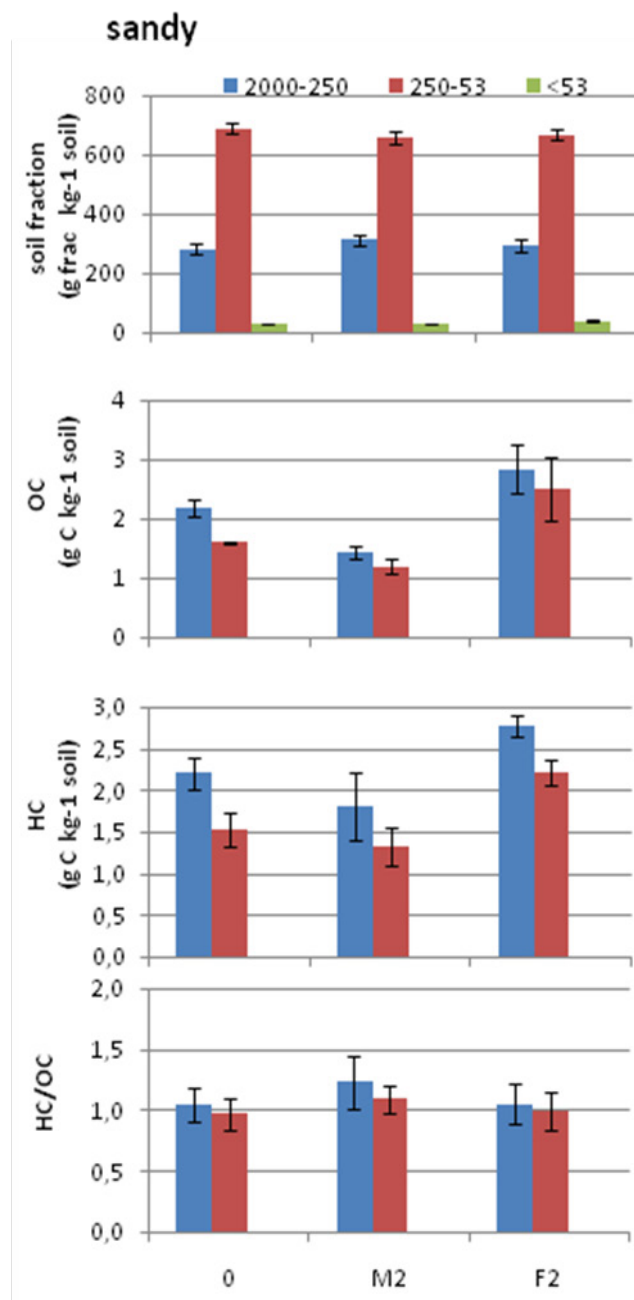


Figure 1c –Sandy soil; Water-stable aggregate distribution (g aggregates kg⁻¹ sand-free soil), CO and HC aggregate concentration (g C kg⁻¹ sand-free aggregate) and HC/CO ratio in the different soils and treatments; macroaggregates 2000-250 μm, microaggregates 250-53 μm. Histograms with the same letters, referring to ANOVA interaction ‘aggregate class’ x ‘fertilization treatment’, are not statistically different at P<0.05. Bars represent the standard error. No sand correction was performed for the sandy soil.



3.2 Characterization of humic material

The molecular weight distributions of extracted humic substances were very similar in the macro and microaggregates of the clay soil (Fig. 2a), where HF2 was dominant and not dependent on the type of fertilization. The control showed the lowest value of HF1, especially in the macroaggregates (7.8%) where it differed from the other fractions. In this treatment, the HF3 fraction was also higher than in M2 and F2. A completely different distribution was observed in the <53 μm fraction. The HF3 was comparable to HF2, whereas HF1 was less than 10% in all treatments.

Molecular weight distribution of the humic fraction in the peaty soil was generally very similar among aggregate classes (Fig. 2b), in which the prevailing HF2 ranged between 65 and 80%. The other two fractions (HF1 and HF3), in general under 20%, resulted in no clear differences depending on fertilization in any aggregate class. On average, HF3 values were 6.9, 13.4, and 20.2% in macro, micro and <53 μm fraction respectively.

In the large fraction (2000-250 μm) of the sandy soil, weight distributions were not significantly affected by fertilization type (Fig. 2c). On average, HF values decreased in the order: HF2 > HF3 > HF1. In the 53-250 μm fraction, manure application significantly increased the humic substances of medium-higher weight (HF2 and HF1) with respect to the same fraction in the O and M2 treatments; on the contrary HF3 was significant higher in the control and mineral treatments.

Figure 2a – Clay soil: distribution of humic substances (HS) apparent molecular weight (%) extracted in the three aggregate sizes separated in the different soils and treatments; HF1: >60 kDa; HF2: 30-60 kDa; HF3: <30 kDa. Histograms with the same letters, referring to ANOVA interaction ‘molecular weight class’ x ‘fertilization treatment’, are not statistically different at P<0.05. Bars represent the standard error.

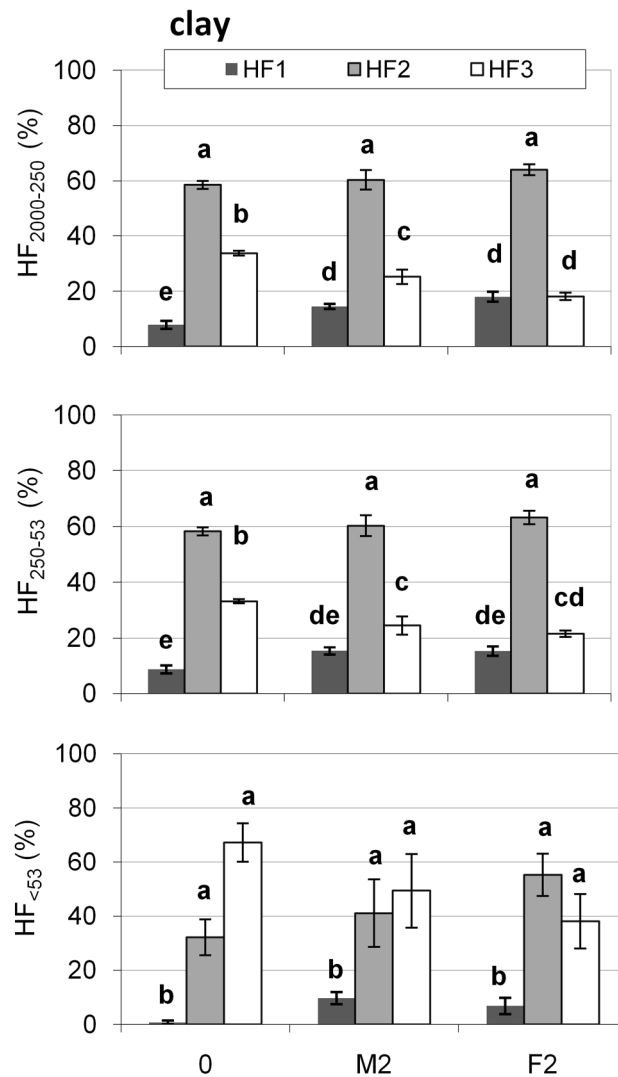


Figure 2b – Peaty soil: distribution of humic substances (HS) apparent molecular weight (%) extracted in the three aggregate sizes separated in the different soils and treatments; HF1: >60 kDa; HF2: 30-60 kDa; HF3: <30 kDa. Histograms with the same letters, referring to ANOVA interaction ‘molecular weight class’ x ‘fertilization treatment’, are not statistically different at P<0.05. Bars represent the standard error.

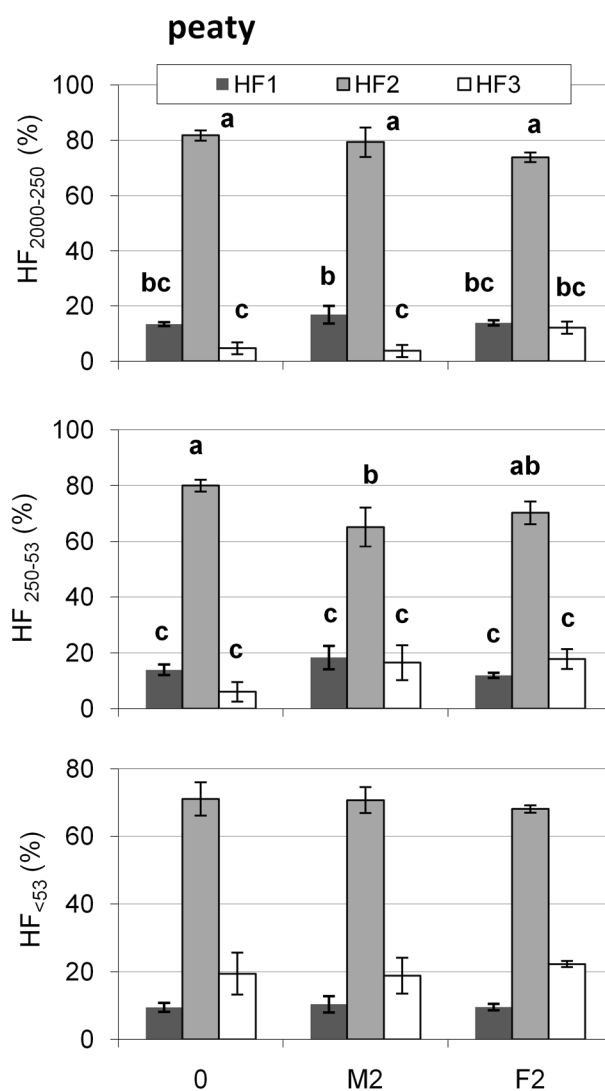
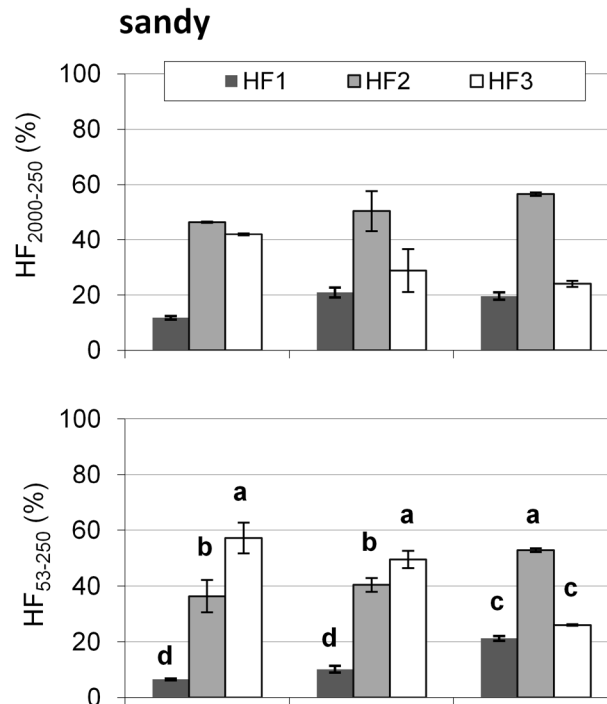


Figure 2b – Sandy soil: distribution of humic substances (HS) apparent molecular weight (%) extracted in the three aggregate sizes separated in the different soils and treatments; HF1: >60 kDa; HF2: 30-60 kDa; HF3: <30 kDa. Histograms with the same letters, referring to ANOVA interaction ‘molecular weight class’ x ‘fertilization treatment’, are not statistically different at $P < 0.05$. Bars represent the standard error.



4. Discussion

The addition of manure significantly increased the proportion of macroaggregates compared with mineral fertilization (Fig. 1a), but only in the clay soil. Mikha and Rice (2004), using a similar N fertilization rate to ours (168 kg ha^{-1}), found a significantly higher proportion of large macroaggregates ($>2000 \mu\text{m}$) in conventionally tilled manured treatments than in the corresponding mineral ones. Gulde et al. (2008) highlighted a greater percentage of the $2000\text{-}250 \mu\text{m}$ aggregate class in the manured treatment (60 t ha^{-1}) than in the control, but with no further increment at higher manuring rate. Inputs of fresh organic material generally promote microbial activity and the release of transient binding agents that could enhance macroaggregation (Six et al., 2004). However, in our experiment, the M2

treatment resulted in the lowest proportion of macroaggregates, whereas the control had an intermediate value (574 g aggr. kg⁻¹ soil), despite receiving less C input (Morari et al., 2006; Lugato et al., 2008). The binding activity of fresh C input may have been offset by the effect of soil porosity distribution during the initial slaking. Indeed, a high volume of large-pores allows a rapid entry of water that causes a build-up of internal air pressure and consequent disruption of the aggregates. Macroporosity volume in the clay soil, as determined in another experiment (unpublished data), was higher in the M2 than O treatment, thus contributing to the aggregate distribution.

The different fertilizations did not affect aggregate distribution in the peaty soil (Fig. 1b), indicating that new organic inputs contribute less to the aggregation process in a soil with a very high SOC content. Moreover, macroaggregate average values were 432 g aggr. kg⁻¹ soil against 575 g aggr. kg⁻¹ soil in the clay soil. These differences could be attributed to the different clay mineralogy (i.e. clay soil has a higher proportion of smectite / montmorillonite), as plant residues, living roots and nutrient additions have been demonstrated to act differently depending on clay mineralogy (Denef et al., 2002; Denef and Six, 2005).

Aggregate hierarchy, according to which SOC concentration increases with increasing aggregate size, was consistent with our results, as in many other studies (Six et al., 2000; Mikha and Rice, 2004; John et al., 2005; Yamashita et al., 2006; Gulde et al., 2008). In the clay and peaty soils the lowest SOC values were measured in the <53 μm fraction and the highest in the macroaggregates (Fig 1a-b), with a significant exception in the microaggregate fraction of the F2-clay soil treatment. Manure application significantly affected the SOC content, particularly in the macro and microaggregates of the clay soil, as also reported by other authors (Mikha and Rice, 2004; Gulde et al., 2008), whereas the <53 μm fraction seemed less affected by the organic application (no significant interaction fertilization x aggregate size in the peaty soil).

The HC values followed the same pattern as the OC, with a very high correlation with this parameter ($r > 0.95$). As a consequence, the HC/OC ratio, in the clay and peaty soil, ranged narrowly among the fertilizations and aggregate fractions without

displaying any hierarchical pattern. In fact, according to the hierarchical concept, humic substances should act as persisting binding agents and be involved in microaggregate stabilization. Our results did not support this theory, as HC/OC was generally homogenous in all the fractions and not higher in microaggregates. Other authors (Bongiovanni and Lombartini, 2006) found higher concentrations of humic and fulvic acid in macro than in microaggregates, in both undisturbed and cultivated soil. They also concluded that these were affected by cultivation to the same magnitude as transient binding agents.

Although HC displayed no hierarchical pattern it was involved in the aggregation process, as noted in particular for the clay soil (Table 2). The WSA was more correlated with the HC than OC, since r values were 0.67 and 0.57, respectively.

Table 2 Correlation matrix of the selected parameters in the clay soil: M = 2000-250 μm macroaggregate proportion; WSA = index of stability; HC-M = humic carbon concentration in macroaggregates; HC = total humic carbon concentration; OC-M = organic carbon concentration in macroaggregates; OC = total organic carbon concentration (values in bold are significant different at P=0.05).

	M	WSA	HC-M	HC	OC-M	OC
M	1.00					
WSA	0.97	1.00				
HC-M	0.45	0.61	1.00			
HC	0.52	0.67	0.98	1.00		
OC-M	0.25	0.40	0.86	0.83	1.00	
OC	0.42	0.57	0.90	0.90	0.97	1.00

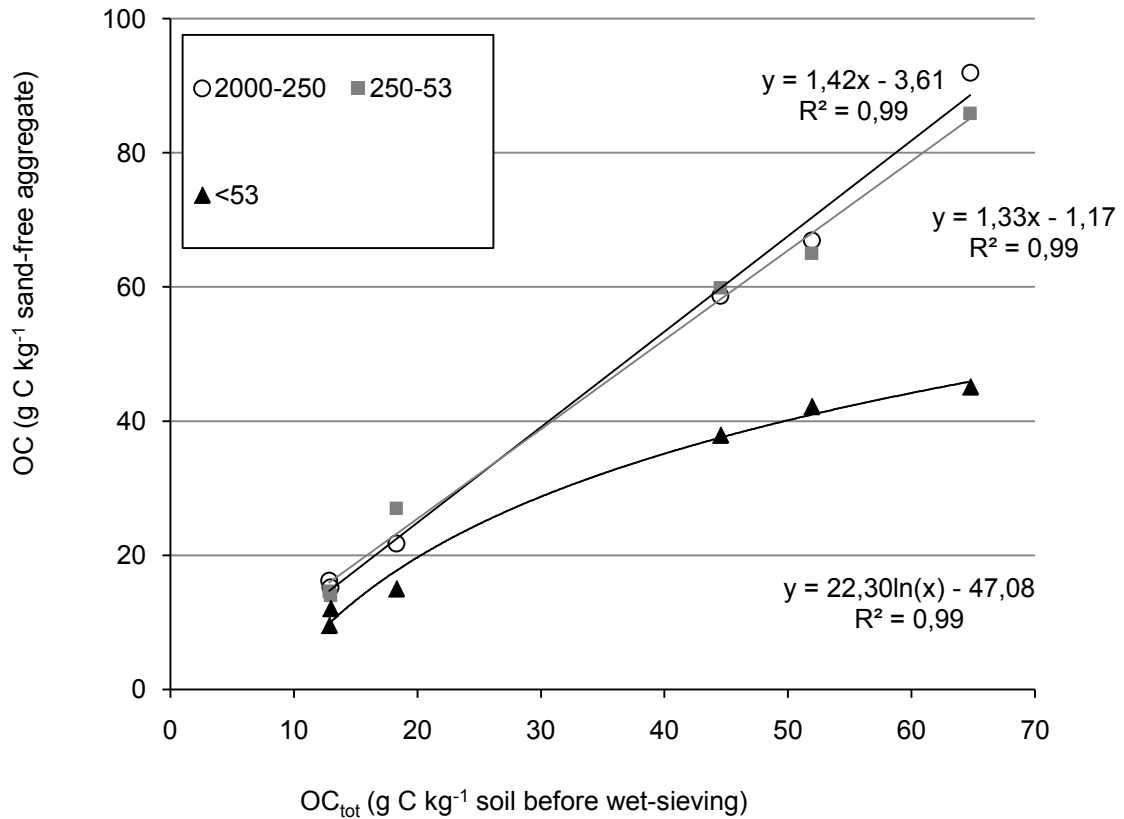
Within the two parameters, the total carbon concentration was therefore more important than the carbon concentration in the macroaggregates (HC-M and OC-M) in influencing the WSA or macroaggregate proportion. The OC and HC not only act as binding agents, but are also effective agents in modifying the entire soil

porosity distribution, as found in previous studies (Lugato et al., 2008), consequently influencing the soil disruption during the slaking.

The HC extracted in clay soil was clearly of different composition depending on the aggregate size. In the <53 μm class (Fig. 2a-b-c), the lightest humic fraction (HF3) prevailed, whereas HF2 reached about 60% in the micro and macroaggregates. To better clarify the chemical composition and possible origin of these substances a more detailed characterization with nuclear magnetic resonance is in progress. However, Mao et al. (2007), analyzing the composition of humic acid (HA) in the particle-size fractions, found a greater proportion of readily oxidizable material in the clay-fraction HA than in the silt-fraction HA. Other recent research suggested that interactions between microbial metabolites and mineral surfaces are important in initiating OM stabilization (Lehmann et al., 2007). The highest value of HF3 in the <53 μm fraction of the clay soil is consistent with these results and the general evidence that clay associated C is enriched by microbial products (Christensen, 2001). These results are confirmed by a previous work (Lugato et al., 2008) investigating aggregate porosity and HS. Indeed, low molecular weight HS were strongly correlated with the crypto porosity domain, where organic matter could interact with clay. HF2 dominated in the peaty soil (Fig. 2b), whereas the average value of HF3 was 20.02% against 6.9% in the <53 μm and macroaggregates. Anyway, HS distribution was the most similar among the aggregate fractions in this soil, leading to the following explanation involving a saturation concept. Recent researches (Gulde et al., 2008; Stewart et al., 2008) have demonstrated that, close to or at C saturation conditions, saturated pools tend to show an asymptotic relationship between whole SOC concentration and SOC concentration for one or more fractions. Our results corroborate these studies, since a deviation from linearity was evident only for the <53 μm fraction (Figure 3) and was determined mainly by the peaty soil behaviour. SOC in the <53 μm fraction has a generally higher mean residence time (i.e. low turnover) (Christensen, 2001), thus it is more difficult for new C to enter this pool, especially when saturated. Consequently, the HC composition in the peaty soil could reflect the presence of “old” carbon that has

not been substituted as in the <53 μm fraction of the clay soil, where unsaturated conditions allow the protection of new carbon.

Figure 3 – Organic C concentration (g C kg^{-1} sand-free aggregate) of the wet-sieved fractions isolated in relation to the total organic C of the sample before wet-sieving in the clay and peaty soils.



We were unable to isolate stable aggregates in the sandy soil, as the lack of clay prevented the formation of primary organomineral complexes and fresh organic input acted only as a very transient binding agent due to the enhanced decomposition conditions (Lugato et al., 2008). Manure affected SOC and HC concentration, but no significant differences emerged between the two size fractions, the OC/HC ratio in particular resulted as very homogenous. However the HC extracted in the larger fraction (2000-250 μm) was in general richer in high molecular weight HS (HS1 and HS2) than the intermediate one (53-250 μm),

especially in the O and M2 treatments, probably due to the higher presence of plant derived C. Manure changed the HS repartition, increasing the high molecular weight % in the 53-250 μm . The same effect was observed in manured treatments of another long-term experiment in a silty soil (Nardi et al., 2004).

5. Conclusions

Our results demonstrated that the addition of manure only significantly increased the proportion of macroaggregates compared to the mineral fertilization in the clay soil, whereas no significant effect was observed in the peaty one. The $<53 \mu\text{m}$ fraction in both soils had a lower SOC concentration than in the micro and macroaggregates, according to the hierarchical concept. HC was not a dominant binding agent at microaggregate level, since the HC/OC ratio was not higher in this fraction. The low expression of this form of C probably indicated that other aggregate agents (soil mineralogy, iron oxides, cations) could play an important role in these soils. The apparent molecular weight of HC was influenced by the aggregate size in which it was extracted and by the manure addition, but its higher homogeneity among aggregate fraction in peaty soil could be related to a saturation mechanism.

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**Chapter III: Qualitative composition of humic substances
in wet-sieved aggregates of different soils**

Introduction

Content and chemical composition of soil organic carbon (SOC) are influenced by management and amendment practices (Venteris et al., 2004; Kaiser and Ellerbrock, 2005). In general, intensive cultivations characterized by conventional soil tillage cause SOC mineralization and depletion (Freibauer et al., 2004) whereas conservative tillage and organic fertilisations (manure and crop-residues) increase the content of SOC and improve its quality (Francioso et al., 2000; Lal, 2004; Nardi et al., 2004; Francioso et al., 2005; Corrado et al., 2008).

The interest in studying organic carbon dynamics in soil is now growing since agroecosystems subjected to various management and amendment practices can have significant implications for carbon sequestration potential in agricultural soils (Lal, 2004; Morari et al., 2006).

The carbon sequestration capabilities of soil depend on the equilibrium resulting from two opposite processes: the first leading the SOC to decomposition with the loss of CO₂, the second to its stabilization. Morphology and the chemical structure of SOC as well as the chemical and physical properties of the mineral matrix affect the processes (Kimetu et al., 2009).

Primary SOC input in agricultural soils consists principally of plant biopolymer residues constituted mainly by polysaccharides (starch, cellulose, hemicellulose and pectin; 50–60%) and lignin (15–20%), but also by proteins, polyphenols (e.g. tannins), chlorophyll, cutin and suberin, lipids and waxes (10–20%) (Lützow et al., 2006). These residues are mainly decomposed by different soil microorganisms, fungi and indirectly by their predators whereas abiotic mineralization is accountable for less than 5% of SOC decomposition (Lavelle et al., 1993). The recalcitrant plant litters compounds and the secondary products resulting from the decomposition processes leads to the formation of different SOC pools that are interested by humification process. Indeed, they are transformed by chemical, biological and physical processes into more stable compounds (Stevenson, 1994; Zech et al., 1997) normally indicated as humic substances (HS). HS are considered to be refractory due to their chemical composition, thus belonging to the refractory SOM pool (Hayes and Clapp, 2001). During humification the amount of aromatic and C-

alkyl carbons increases whereas the level of O-alkyl carbon decreases (Baldock et al., 1991; Guggenberger et al., 1995; Kögel-Knabner, 1997; Shang and Tissen, 1998; Six et al., 1998; Chefetz et al., 2000) probably due to the microbially mediated consumption of O-alkyl carbons (i.e., carbohydrates). This cause a relative increase of the more refractory SOC fractions (i.e., aromatic and alkyl structures). The application of organic fertilizers plays a pivotal role in SOC dynamics. For instance, Nardi et al. (2004) observed as long-term application of farmyard manure directed the turnover of SOC towards the humification process with a high-quality humus production, higher degree of policondensation and resistance to microbial attack.

While chemical composition could influence the rate of decomposition and/or stabilization of SOC, its associations with soil minerals and distribution between different physical locations in the soil matrix must also be a controlling factors.

Aggregation is considered a key role factor in SOC protection because occluded organic matter is spatially protected against decomposition due to: reduced access for the microorganisms and their enzymes; reduced diffusion of enzymes into the intra-aggregate space; and restricted aerobic decomposition caused by reduced diffusion of oxygen (Lützow et al., 2006).

By applying a combination of physical fractionation and chemical characterization, it should therefore be possible to identify fractions that are dominated by a particular mechanism of protection. A common approach is the slaking and wet sieving fractionation technique that aims to isolate stable aggregate classes (macroaggregates 250-2000 μm , microaggregates 53-250 μm and silt-clay <53 μm) (Six et al., 1998).

Macroaggregates often contain higher SOC content than microaggregates, because the former include microaggregates plus coarse organic matter serving as an intra-macroaggregate binding agent (Cambardella and Elliot, 1993; Jastrow et al., 1996; Puget et al., 1995; Six et al., 2000). The turnover of OM in macroaggregates is much faster than in the smaller ones (Six et al., 1998; Six et al., 2002; John et al., 2005) and for this reason they seem not accountable of protection by spatial exclusion.

Many authors applied ^{13}C NMR and infrared spectroscopy for the study of SOC composition in the different soil fractions and HS substances. They recognize that SOC into macroaggregates is mainly composed by lignin and O-alkyl macromolecules, plant lipids, chitin, melanin, suberin, microbial polysaccharides and long-chain straight hydrocarbons; these compounds resist to decomposition mainly because of their chemical composition. The aliphatic portions of cutin and suberin are particularly resistant to decomposition (Huang et al. 1999)

Soil microaggregates are considered very stable due to their chemical-physical genesis and different physical properties; for this reason they play a key role in SOC conservation. They are assumed to be stabilized by organic persisting binding agents, in particular mature HS predominately formed by persistent lignin components, fats and waxes (Waksman, 1938; Umbreit, 1962).

The silt clay fraction is characterized by a high level aliphatic compounds (alkyl-C). The main protection processes identified for this fraction are the interactions of OM with the mineral surfaces and the intercalation of OM into the interlayer spaces of expandable phyllosilicates (2:1 clays). Sorption occurs via a variety of organomineral associations, such as polyvalent cation bridges, hydrogenbonding, van der Waals forces, and interactions with hydrous oxides and aluminosilicates (Jastrow et al., 2007). Mahieu et al. (1999) and Chen et Chiu (2003) reported that the abundance of alkyl C in soil organic matter increased systematically with decreasing particle size. Lützow et al. (2006) reports an estimated OM turnover in the silt clay fraction more than 100 years.

As consequence functional groups of organic and HS substances are strongly dependant to the different soil fractions and their corresponding mechanisms of stabilization.

Moreover only few researches have investigated the HS role and composition in the different soil aggregate fractions and how strategies for sequestering C in the soil (e.g. organic fertilizers) can affect them in the long-term period. Indeed, SOC changes very step by step and many years are required to measure the structural variation in the soil (Pascual et al., 1999).

The objective of this work was to study in a long-term experiments a) how the main chemical HS compounds are dependent on the different aggregates classes and b) the effects of long-term (44-yrs) organic fertilisation applications on SOC stabilization mechanisms.

Material and Methods

2.1 The long-term experiment

The long-term trial is located at the Experimental Farm of the University of Padova (Veneto Region, NE Italy). The local climate is sub-humid, with annual rainfall of about 850 mm and yearly average temperature of 12 °C. The reference evapotranspiration (ET_o) is 945 mm with a peak in July (5 mm d⁻¹). ET_o exceeds rainfall from April to September. The site has a shallow water table ranging from about 0.5-1.5 m in late winter-early spring to 1-2 m in summer.

This experiment began in 1964 in 4 m² open lysimeters, 80 cm deep. The experimental treatments derive from the factorial combination of three types of soil (herein after called clay, sandy and peaty in relation to their dominant property in Table 1) with six types of mineral, organic or mixed fertilization, organized in two randomized blocks (36 lysimeters).

The soils were brought from three locations in the Veneto region: clay soil from the south-western plain, sandy soil from the central coastal area and peaty soil from the southern plain. The original soil profiles were reconstructed in the lysimeters. The sandy soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) contain predominantly quartz and feldspar and a significant amount of dolomite (16 %). Clay soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) have the higher amount of smectite / montmorillonite (16 %) than the other soils and a considerable presence of mica (19%) and dolomite (15 %). The peaty soils (Typic Sulphisaprists euic, mesic – ARPAV, 2005) are characterized by a higher content of mica (25 %) whereas smectite / montmorillonite reach the 12 %. After 40 years of experimental conditions, some

original properties are changed as reported recently in Morari et al., (2008) and in table 1.

Fertilization treatments considered for this study were as follows: no applications (O); farmyard manure – F2 (40 t ha⁻¹ yr⁻¹); mineral fertilizer – M2 (200 kg ha⁻¹ yr⁻¹ N - 100 P₂O₅ - 240 K₂O). The F2 applied fairly the same amount of macroelements than M2 and about 3.5-4 t C ha⁻¹ y⁻¹. Until 1984 there was a two-year maize (*Zea mays* L.) - wheat rotation (*Triticum aestivum* L.). Thereafter, a variable rotation was adopted between 1985 and 1992, with various horticultural crops. Since 1993 there has been a three-year rotation of tomato (*Lycopersicon esculentum* Mill.) – sugarbeet (*Beta vulgaris* L.) – maize, followed by various horticultural crops, maize and sunflower (*Helianthus annuus* L.) from 2003 to 2007. Apart from fertilization, all plots were treated in the same way in terms of rotation and management (tillage, sowing, harvest, etc). The top 15-20 cm was dug each autumn and crop residues were removed from all treatments.

2.2 Soil sampling and fractionation technique

The top soil layer (0-20 cm) was sampled in June 2008. The samples were taken from five different points in the whole lysimeter area and bulked to obtain a sample of about 1 kg. Samples were broken up manually, air-dried and sieved at 2 mm. To standardize the fractionation procedure and better evidence the relationship between aggregation and organic binding agent in the different soils, we first isolated the large macroaggregates (from 1 to 2 mm) by dry-sieving. More than 50-60% of the total mass was accounted in the 1-2 mm aggregate fraction. No large aggregates were isolated from sandy soil, so all the soil was retained. Large aggregates (two sub-samples per plot - 36 in total) were then separated into three aggregate size fractions by wet-sieving, using an automatic machine oscillator. Briefly, the apparatus produces vertical actions which transmit a 3 cm stroke vertical movement to a stack of interfitting sieves with a mesh of 250 and 53 µm. The sieves, 10 cm in diameter and 5 cm height, were immersed in distilled water inside a beaker (15 cm diameter x 18 cm height). Prior to sieving, the water level was adjusted to prevent water reflux over the edge of the 250 µm sieve during the oscillation.

Table 1 Physical-chemical characteristics of the 0-30 cm depth at the beginning of the experiment (1964).

Parameters	clay	sandy	peaty
Sand (2 mm –50 μm) (%)	25.0	93.4	38.0
Silt (50-2 μm) (%)	23.0	6.0	13.6
Clay (<2 μm) (%)	52.0	0.6	48.4
pH	7.9 (7.7)	8.1 (7.7)	4.9 (7.2)
Total Carbonate (g kg^{-1})	26.0	39.0	0.0
Organic Carbon (g kg^{-1})	14.5 (15.7)	1.7 (3.1)	105.0 (89)
Total Nitrogen (g kg^{-1})	1.5 (1.5)	0.15	6.7 (6.5)
C/N	10.0 (10.2)	12.0	16.0 (13.6)
Total Phosphorus (g kg^{-1})	2.8 (5.6)	0.5 (1.8)	1.1 (3.1)
Available Phosphorus (mg kg^{-1})	161.5	26.2	100.4

Values between brackets were measured in 2002

Before sieving, 15 g of sample were immersed on top of the 250 μm sieve for 10 min to allow slaking. After slaking, aggregates were separated by vertical oscillations at a frequency of 50 rpm for 18 min (for a total of 900 oscillations). Macroaggregates (2000-250 μm) and microaggregates (53-250 μm) were then collected from the corresponding sieves, whereas the silt-clay fraction (<53 μm) remaining in the beaker was precipitated by adding 3 ml of 1N CaCl_2 . In sandy soil the silt-clay fraction was not recovered due to its very low amount and in general, due to the lack of aggregation, we can define macroaggregates (2000-250 μm) and microaggregates (53-250 μm) as coarse and fine sand respectively. All isolated fractions were dried at 60 °C in a forced-air oven.

2.3 Humic carbon extraction

The humic carbon of the different aggregate fraction (WSA) were extracted from the air-dried samples with 0.5 M NaOH (1:10 w/v) in Dubnoff bath at 50 °C for 16 h and were separated from the suspended material by centrifuging at 15000 rpm for 15 min. Here, the term humic substances is the fraction soluble in bases and comprehensive of humic and fulvic acids. Humic extracts (50 ml) were transferred into 18,000 mol. wt cut-off dialysis Visking tubing (Medicell Ltd., London, UK) and dialyzed against double-distilled water. The water was changed daily until the liquid outside the dialysis tube was colorless. Moreover, the samples were treated with an ion exchange on Amberlite IR 120 H⁺ (Stevenson, 1994) to remove other cations. Finally the humic extracts were dried by lyophilizing before spectroscopic analyses. Prior to perform chemical analysis HS extracted from the two replicates were mixed together to form a single sample. In total 24 HS samples were analyzed (3 soils X 3 treatments X 3 aggregate fractions minus 3 silt-clay fractions in sandy soil).

2.4 CN determination

HS total C and N were analyzed on a CNS automatic analyzer (Vario Macro, Elementar). The basic principle of operation is high temperature digestion of 200 mg HS at 800°C to 1200°C with subsequent scrubbing of non-analytes from the combustion gases. The analyte gases are transported in helium carrier stream. After reduction of formed nitrogen oxides the gas mixture is separated in its components which are sequentially released to a detector (TCD) and determines C and N content. Furthermore, percent contents of the elements are calculated from the detector signal in connection with the sample weight and the stored calibration curve.

2.5 Solid state ¹³C-nmr

Solid state ¹³C NMR characterization was performed on a Bruker Avance 400 WB spectrometer equipped for solid state analysis and operating at 100.61 MHz. Samples were spun at 12.5 kHz in 4 mm diameter zirconia rotors with Kel-F caps.

The ^{13}C SPE (SPE=single pulse experiment) MAS NMR spectra were obtained with high power proton decoupling during acquisition, 30 seconds relaxing delay (D1), 3072 scans (NS) , and processed with a 100 Hz exponential line broadening. ^{13}C chemical shifts were externally referenced to solid sodium 3-(trimethyl-silyl)-1-propane sulfonate at 0 ppm. Magic angle conditions were adjusted by observing ^{79}Br spinning side bands pattern in a rotor containing 5% of KBr.

It is noteworthy that collected spectra reveal the presence of a several kHz wide undesired band, which is superimposed to the resonances assigned to humic substances. That wide band is due to the presence of carbon atom inside the probe and rotor cap materials. For this reason, the ^{13}C SPE MAS NMR spectrum of an empty rotor was recorded by employing the same acquisition parameters above described and then subtracted from each measurement collected in presence of sample: the obtained ^{13}C SPE MAS NMR spectra were employed for quantitative analysis of humic substances. All molecular moieties detectable in samples were grouped into five main chemical functionality classes. Each class was quantified by integrating resonance signals about the following ppm ranges: 182-158 (carbonyl c), 158-108 (aromatic c), 108-90 (acetal c), 90-35 (O, N-alkyl), 35-5 (alkyl c).

2.6 Diffuse reflectance infrared Fourier transform spectroscopy

DRIFT spectrum was recorded with a Bruker TENSOR series FT-IR spectrophotometer (Ettlingen, Germany) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). The spectrum was collected from 4000 to 400 cm^{-1} and averaged over 64 scans (resolution 4 cm^{-1}) and converted into Kubelka-Munk units. KBr powder (Aldrich Chemical Co. Milwaukee, WI) was used for the background reference. Analyses of spectral data were performed with Grams/386 spectral software (Galactic Industries, Salem, NH). We decided to explore only the extracted SOM instead of single soil fractions because the spectra of soil fractions were strongly influenced by the intense signal of mineral component (data not reported).

Results and Discussion

3.1 Chemical analyses

The humic C of untreated clay soil (Table 2) was differently distributed between aggregate size fractions resulting less than 3% and 4% in micro and silt-clay fractions, respectively. Similarly the N content progressively decreased of 2.7% in both finest fractions. After 40 yrs of inorganic fertilization no significant change was observed between macro and silt-clay fractions while the amount of C and N respectively fell off 2.4% in microaggregate size fractions. The long term manure amendment did not cause apparent changes between macro and microaggregate size fractions whilst the amount of C was less than 1.8% and N content increased of 3 % in silt-clay fraction.

In general the amount of humic C in macroaggregate size fractions seem to be independent by treatment kind. Instead the effect of treatments on humic C and N content was well spotlighted in microaggregate and silt clay fractions. The inorganic fertilization only caused a considerable increased of humic C (above 5%) in silt-clay fraction. Moreover, an apparent accumulation of humic C due to the effect of amendment with manure was observed in micro (+3.8%) and silt-clay (+2%) fractions, respectively. In regard to N content a positive effect of inorganic fertilization was estimated in the silt clay fraction (+2.4%) while an unexpected loss of 7% in microaggregate and 4.7% in silt clay fractions was observed as a consequence of manure treatment. The C/N ratio evidenced lower values and consequently an apparent increase of humification processes in silt-clay fractions of peaty and in FMY treatment of clay soil.

Table 2: Total C and N content and C/N ratio in humic substances extracted from different aggregate size-fractions of clay, peaty and sandy soils treated consecutively over 40 yrs with mineral fertilizers (MIN) and manure (FMY)

	UNTREATED			MIN			FMY		
	C	N (%)	C/N	C	N (%)	C/N	C	N (%)	C/N
Clay									
>250	45.4	5.44	8.3	45.2	5.43	8.3	45.3	4.89	9.3
53-250	44.0	5.29	8.3	44.1	5.30	8.3	45.7	4.91	9.3
<53	43.6	5.29	8.2	45.9	5.42	8.5	44.5	5.04	8.8
Peaty									
>250	46.1	3.84	12.0	46.9	3.91	11.9	45.0	3.94	11.4
53-250	46.0	3.91	11.7	45.7	3.88	11.7	47.12	4.01	11.74
<53	46.5	4.50	10.36	45.4	4.22	10.7	46.98	4.28	10.98
Sand									
>250	39.6	2.60	15.2	43.2	2.33	18.5	46.8	3.74	12.5
53-250	43.9	2.71	16.1	nd.	nd.	nd.	45.7	4.04	11.3

3.2 ¹³C SPE MAS NMR spectroscopy

The NMR spectra of the HS from the untreated clay showed a similar pattern between the aggregates (Figure 1a). However, the main change between the spectra were assigned to quantitative distribution of ¹³C between fractions (Table 3).

The HS of macroaggregate size fraction showed the highest level of O, N-alkyl/methoxy (55% Table 3) probably derived by plant residues such as hemicellulose and cellulose and a smaller proportion of aromatic C (~5% Table 3). On the contrast the HS spectrum of micro-aggregate displayed a higher levels of

aromatic C (~6% Table 3) and carbonyl C (~20% Table 3). This aggregate-size-fraction is considered to be sensitive to land-use and management effects (Six et al., 1998). A decrease of O, N alkyl C from microaggregate size to silt-clay fraction together with an increasing proportion of alkyl-C is indicative of decomposition process of organic matter in soils (Leifeld et al., 2002). Several studies have shown that high level of alkyl C arise from plant residues and can be preserved in soils with little or no alteration (Almendros et al., 1996). Thus, these compounds can be easily accumulated and included in the HS structure (Zech et al., 1997) through hydrophobic interaction (Piccolo et al. 2002) and to be stabilized in the silt-clay structure. A larger proportion of anomeric C (106 ppm) in silt-clay might also be originated by microorganisms as discussed by Baldock and Skjemstad (2000). They found in the fine clay of soil a large proportion of polysaccharide and smectite. The fact that polysaccharides, considered labile compounds, are accumulated in this fraction is probably due to close physical-chemical interactions with clay surface and as a consequence the polysaccharides are protected against decomposition. After 40 yrs of mineral fertilization (Figure 1a) the O, N-alkyl-C content of HS decreased from macro-aggregate (54 % Table 3) to silt clay (50% Table 3) fractions whereas the alkyl-C content increased. However, the HS of microaggregate size fraction had the higher proportion in O, N alkyl C (~20%) and more recalcitrant alkyl-C indicating that plant residues are not so extensively decomposed, evidencing selective preservation due to chemical composition of alkyl-C recalcitrant compounds. This can also be supported by low proportion of aromatic C found in this sample. The HS of silt-clay fraction showed a highest amount in aromatic C (~7% Table 3) and carbonyl C (~21% Table 2) which was approximately more concentrated than to that of respective fraction of untreated sample. Carbonyl C compounds can be efficiently protected by spatial inaccessibility processes. We find that the HS structure did not seem be considerably modified by inorganic fertilization apart from slight increase in aromatic and carbonyl C in silt-clay fraction. The long-term of the application of FMY (Figure 1a) decreased the O, N-alkyl-C content and alkyl C and an increase in aromatic and carbonyl C from macro to

micro-aggregates-size fractions (Table 3). The proportion of aromatic C in the micro-aggregate-size fraction might be favoured by a high metabolic activity of microbial biomass as a consequence of a greater availability of readily biodegradable organic matter. In addition specific surface area provided by clay mineral may stabilize microbial metabolites produced from decomposing plant (Saggar et al., 1996). Both factors can promote the selective preservation and relative accumulation of recalcitrant aromatic C. This is also qualitatively supported by the presence of phenolic compounds (150 ppm) that were not shown in the untreated and mineral spectra. There was no clear difference between the portion of the alkyl-C and aromatic C in the macroaggregate size and silt-clay fractions. The anomeric-C did not seem to be influenced by the manure treatment instead it decreased with respect to untreated and inorganic fertilization aggregate-size-fractions. This significant variation is probably related to an intensification of microbial biomass activity as a consequence of manure treatment.

Our results confirm the literature data on positive effect of long-term manuring on the chemical composition of HS (Mao et al., 2008, Senesi et al., 2007, Brunetti et al., 2007).

The NMR spectrum of the HS from the untreated peat (Figure 1b) showed a progressive decrease of proportion in alkyl-C and O-N alkyl-C between the aggregates fractions (Table 3). On the contrast, the proportion of aromatic C increased from macro-aggregate-size to silt-clay fractions. The different accumulation in silt-clay fraction of aromatic C together to that of carbonyl C might correspond to a high humification rank. In fact the low proportion of anomeric C, despite of the continuous input of organic C from roots and their exudates, gives an indication of the degree of polysaccharide decomposition (Schlecht-Pietsch et al., 1994).

Figure 1a: CPMAS ¹³C NMR spectra of HS from different clay aggregate size fractions

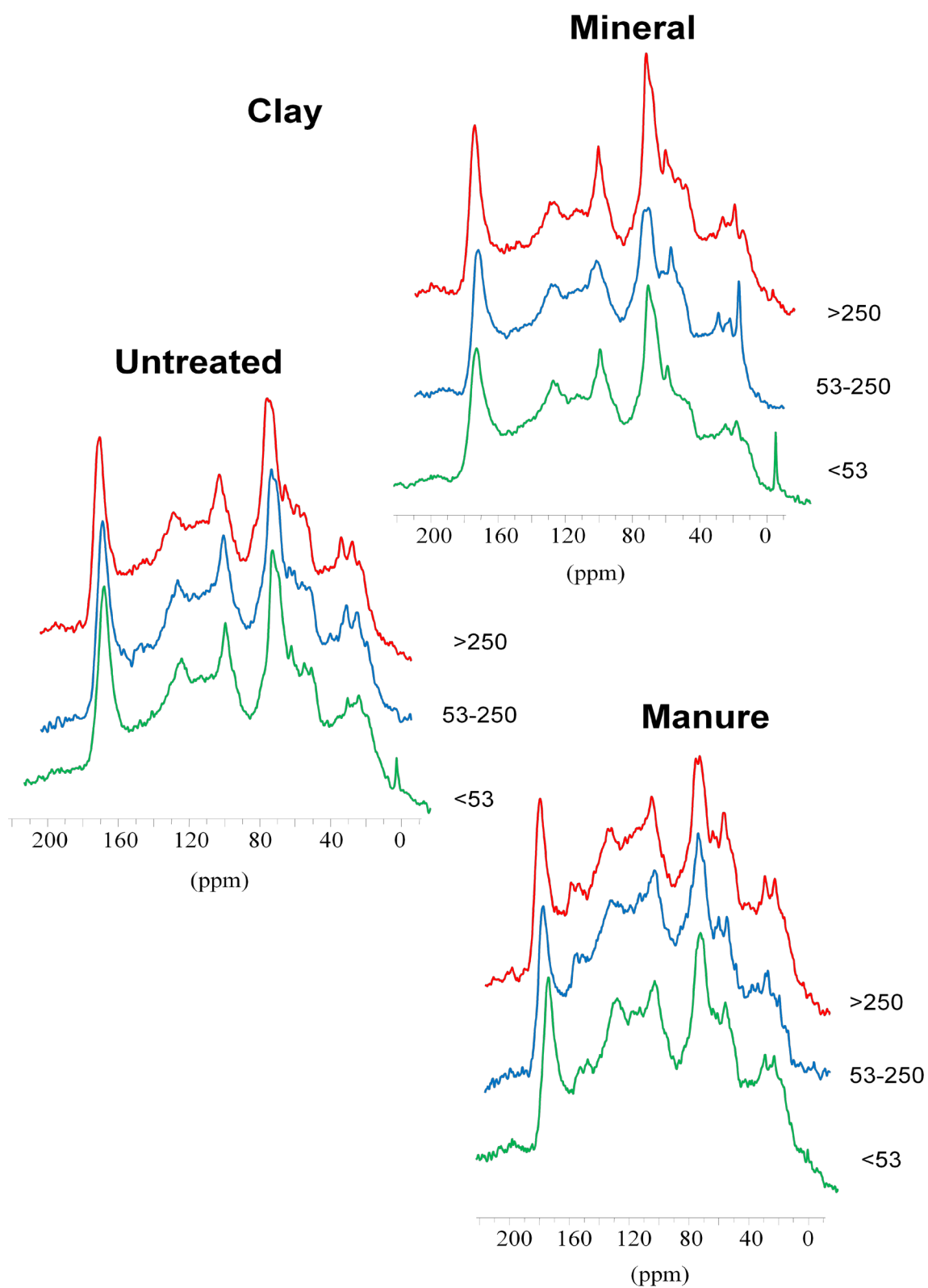


Figure 1b: CPMAS ¹³C NMR spectra of HS from different peat aggregate size fractions

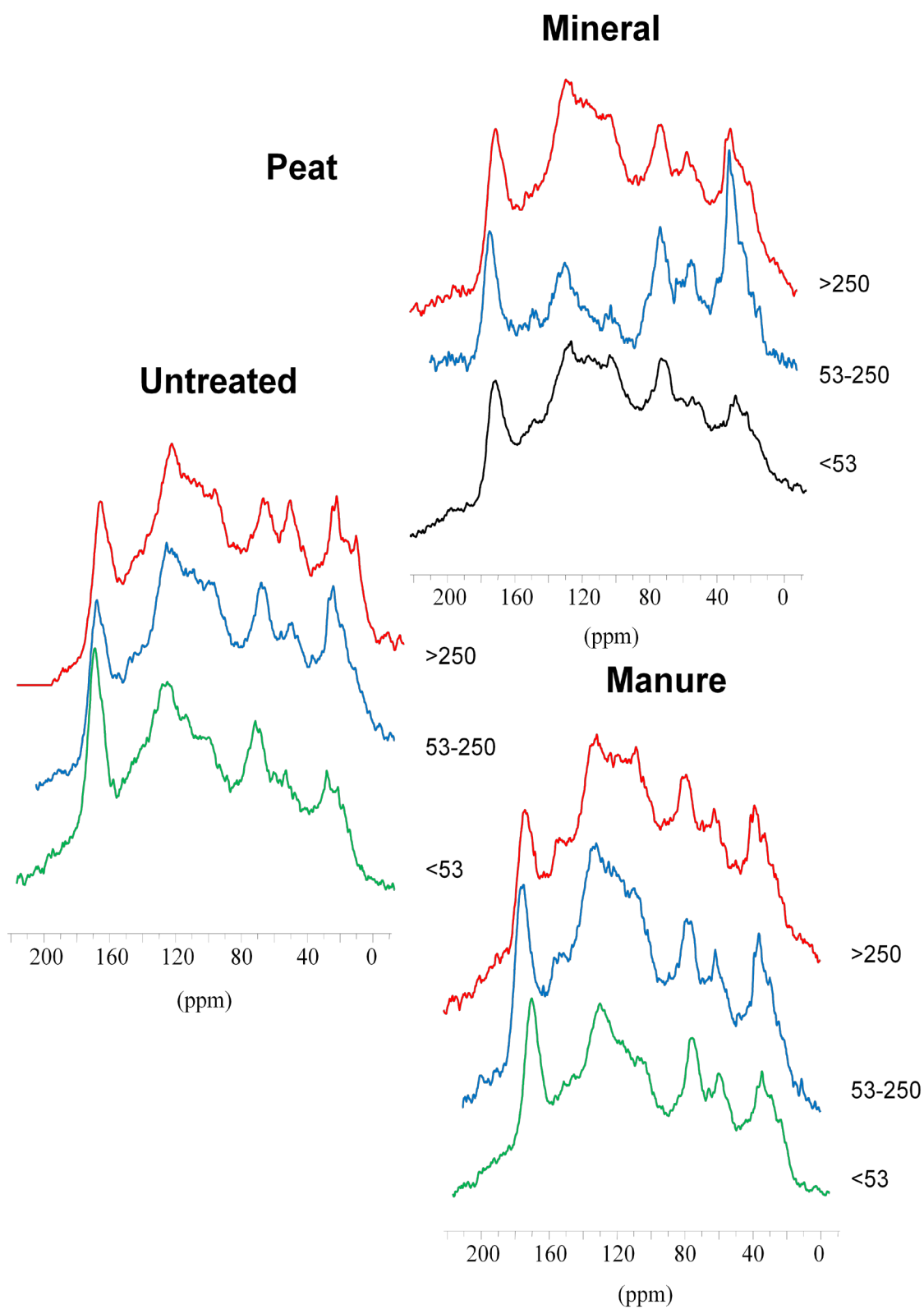


Figure 1c: CPMAS ¹³C NMR spectra of HS from different sand aggregate size fractions

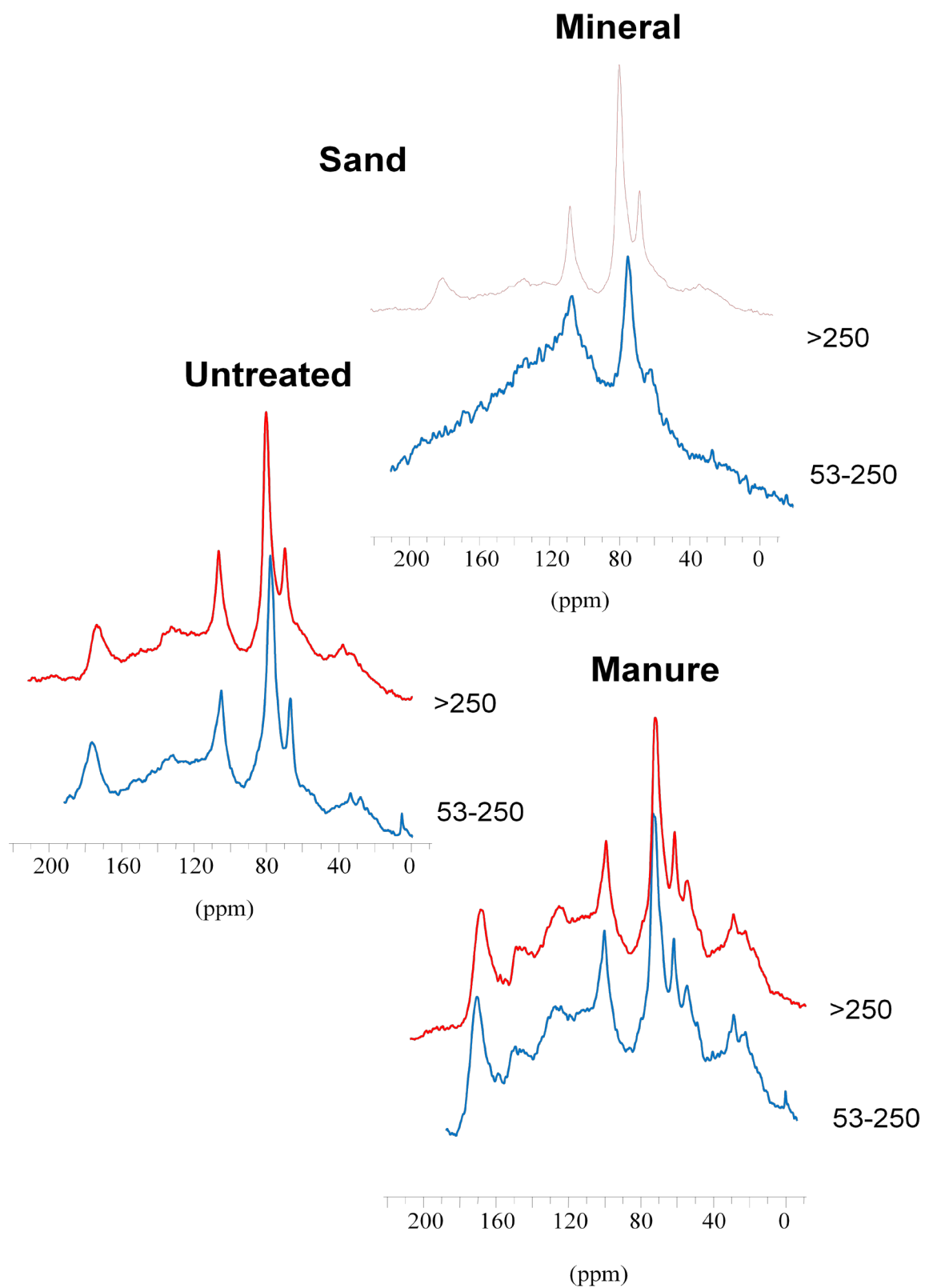


Table 3: Integration area (in percentage) of different ¹³C resonances of HS

(%)	Alkyl- C	O, N Alkyl-C	Anomeric-C	Aryl-C	Carbonyl C
Clay					
Untreated					
>250	16.53	55.03	4.90	5.11	18.43
53-250	16.48	52.89	4.56	6.45	19.62
<53	20.31	50.24	5.57	5.41	18.47
Mineral					
250	13.63	54.00	5.46	5.57	18.34
53-250	19.97	54.46	4.27	4.29	17.11
<53	16.56	50.12	5.74	6.28	21.30
Manure					
250	20.05	53.96	3.62	6.91	15.46
53-250	11.84	51.65	3.82	12.06	20.62
<53	19.59	51.57	3.53	6.74	18.47
Peat					
Untreated					
250	20.91	37.33	1.70	24.24	15.81
53-250	17.30	31.54	2.53	30.38	18.25
<53	11.26	25.31	1.64	35.34	26.45
Mineral					
250	17.31	29.23	3.16	33.26	17.04
53-250	27.84	35.08	1.60	21.25	14.24
<53	19.63	42.65	2.55	16.84	18.32
Manure					
250	15.27	31.85	3.41	32.97	16.50
53-250	15.12	26.01	4.27	37.98	16.62
<53	11.64	24.58	nd	38.54	25.24
Sand					
Untreated					
250	6.55	67.21	11.65	4.45	10.14
53-250	8.75	60.79	12.10	7.11	11.24
Mineral					
250	9.43	68.8	11.39	3.86	6.55
53-250	2.35	63.34	16.83	10.26	7.22
Manure					
250	14.42	56.32	6.13	10.25	12.88
53-250	16.92	50.45	7.35	11.67	13.61

Chemical fertilization significantly changed the trend of O, N alkyl C and alkyl C proportions with respect to untreated aggregate-size fractions. O, N-alkyl C and alkyl C content progressively increased from macro aggregate-size to silt-clay

fractions and whereas the aromatic C decreased. Particularly relevant was the ratio alkyl C/O, N alkyl C in microaggregate size fraction. It was the unique fraction with the value that tended to 1. Usually this ratio increases markedly with an increasing extent of degradation of the organic material hence we can suppose a high humification rank of this HS. The unexpected high proportion of O, N alkyl C (~43% Table3) in silt-clay fraction gives an idea of recent formation of humic C, as also supported by low content of aromatic C(~17% Table 3). Thus, the HS of silt-clay fraction can be in a transitional stage in the humification process. On the contrary, the HS spectra profile of FMY treatments, resulted similar to those of untreated aggregate-size fractions. However, the amount of alkyl C and O, N alkyl C progressively decreased from macro aggregate-size to silt-clay fractions while the amount of aromatic C and carbonyl C progressively increased. The presence of phenols (~150 ppm) was only found in macro and microaggregate size fractions but no resonance was observed in untreated and mineral fertilization aggregate-size fractions. The phenolic compounds in these fractions can be explained by the incorporation of cereal straw, supplied with manure. Moreover highest levels in O, N-alkyl C and anomeric C were found in these aggregate-size fractions. This general trend, in accordance with the results shown by Tarchitzky et al. (2000), suggested that the HS composition of macro-aggregate size fractions can preserve features very close to that of the plant residues. On the basis of these results decomposition and humification mechanisms of organic matter seem to be related to aggregate size.

NMR spectra of sandy soil fractions were shown in Figure 1c. A considerable amount of O, N-alkyl-C and anomeric C was detected for all samples. The relative enrichment in carbohydrates in the sand fractions may reflect the presence of undecomposed organic matter, rich in cellulose and hemicellulose (Turchenek and Oades, 1979). This may depend on the decrease in microbial-derived carbohydrates and an enrichment in plant-derived carbohydrates (Jolivet et al., 2006). In general it corresponded to about 70% of the total ^{13}C . The O, N-alkyl-C content of macro-aggregate (67%, Table 2) was higher than that of the micro-aggregate size fraction (~60 %), and the alkyl and the aromatic-C contents were

lower (Table 3). Only a slight increase in refractory C such as aromatic and alkyl-C (Table 3) differed in both aggregate size fractions. After 40 yrs of inorganic fertilization no significant structural modification was observed.

On the contrast, FMY improved quali-quantitatively the HS composition. The HS of macro and micro aggregate-size-fractions consisted mainly of O, N-alkyl-C which ranged from 56 to 50% and alkyl-C which ranged from 14% to 17%, respectively. The carbonyl-C contents ranged from 13 to 14 % and the aromatic-C contents varied between 10 and 12%, respectively. Moreover, in the spectra appeared two new bands assigned to O-CH₃ signals (at around 55 ppm) and carbons bonded to phenolic OH (at around 155 ppm) (Sierra et al., 2005). These types of bands seem to be characteristic of lignin (Mao et al. 2008).

The lack of a consistent structural modification in both aggregate size fractions demonstrated the important role of spatial inaccessibility protection processes, like aggregation and interaction with clay mineral surfaces, in decomposition and humification processes of organic matter (Lützow et al, 2006).

A clear structural difference with respect to untreated fractions was observed. The main chemical changes were characterized by a loss of carbohydrates and a concomitant increase in aliphatic, aromatic components. The significant enhancement in alkyl C might be related to accumulation of fatty acids present naturally in manure (Mao et al., 2008) or an intensification of biomass biological activity that is often associated with manure application (Albiach et al., 2000; Fliebbach et al., 2007) rather than to an increase of humification rank.

3.3 DRIFT spectroscopy

The DRIFT spectra of HS samples from different soil aggregate size fractions exhibited a similar spectral pattern among treatments (Fig. 2a,2b,2c). The spectra were interpreted on the base of literature (Olk et al., 2000; Anelli et al., 2000; Tan 2003; Montecchio et al., 2006; Francioso et al., 2007; Francioso et al., 2008; Mao et al., 2008).

The spectra were characterized by following absorbance bands (Table 4): a broad band around 3400-3300 cm⁻¹ (OH stretch of different groups), a shoulder at 3100-3040 cm⁻¹ (NH₃⁺ stretch and C=CH stretch vibrations), the peaks at around 2960

and 2870 cm^{-1} and 2930 and 2940 cm^{-1} (CH_3 and CH_2 stretch of aliphatic chains, respectively), a broad band at $2600\text{-}2500\text{ cm}^{-1}$ (OH stretch in dimeric acids), a strong bands at around $1710\text{-}1660\text{ cm}^{-1}$ ($\text{C}=\text{O}$ stretch of carboxylic acids), an intense band at around 1650 cm^{-1} (amide I, $\text{C}=\text{C}$ stretch vibration), a medium peak at around 1560 cm^{-1} (amide II, aromatic rings), a weak peak at around 1450 cm^{-1} (CH_3 asymmetrical bending and CH_2 scissoring vibrations), a shoulder at around 1415 (CH_2 scissoring vibration of CH_2 adjacent to carbonyl group), a variable intensity peak between $1230\text{-}1250\text{ cm}^{-1}$ (amide III, C-O stretch of aromatic rings and carboxylic acids), a strong peak between $1100\text{-}1030\text{ cm}^{-1}$ and minor absorption peak at 900 cm^{-1} (C-O stretch of cellulose and other β - anomers and β -glycosides).

HS spectra from untreated clay aggregate-size fractions (Figure 2a) showed only slight modifications relative to carbohydrate region ($1100\text{-}1050\text{ cm}^{-1}$) and carbonyl group (1722 cm^{-1}). In macroaggregate size fraction the carbohydrate region appeared more broad than to other size fractions suggesting a more complex structure of carbohydrates while no variation was observed between micro and silt-clay fractions. Moreover, the relative intensity of the peak at 1722 cm^{-1} slightly increased in silt-clay fraction. After 44 yrs of inorganic fertilization the main change regarded the relative intensity of carbonyl group (1712 cm^{-1}) and C-O group (1234 cm^{-1}). The first one gradually increased in micro and silt-clay fractions with respect to macroaggregates. C-O group markedly increased in micro aggregate-size fraction, with a relevant shift of this band to low frequencies from macro to silt-clay fraction. A negative shift of -12 cm^{-1} for microaggregate and -17 cm^{-1} for silt clay fractions might indicate the presence of C-O group in carboxylic acids rather than to C-O in phenols (Rao, 1963) (support NMR).

Figure 2a: Clay soil; DRIFT spectroscopy spectra of HS from different aggregates size fractions

CLAY SOIL

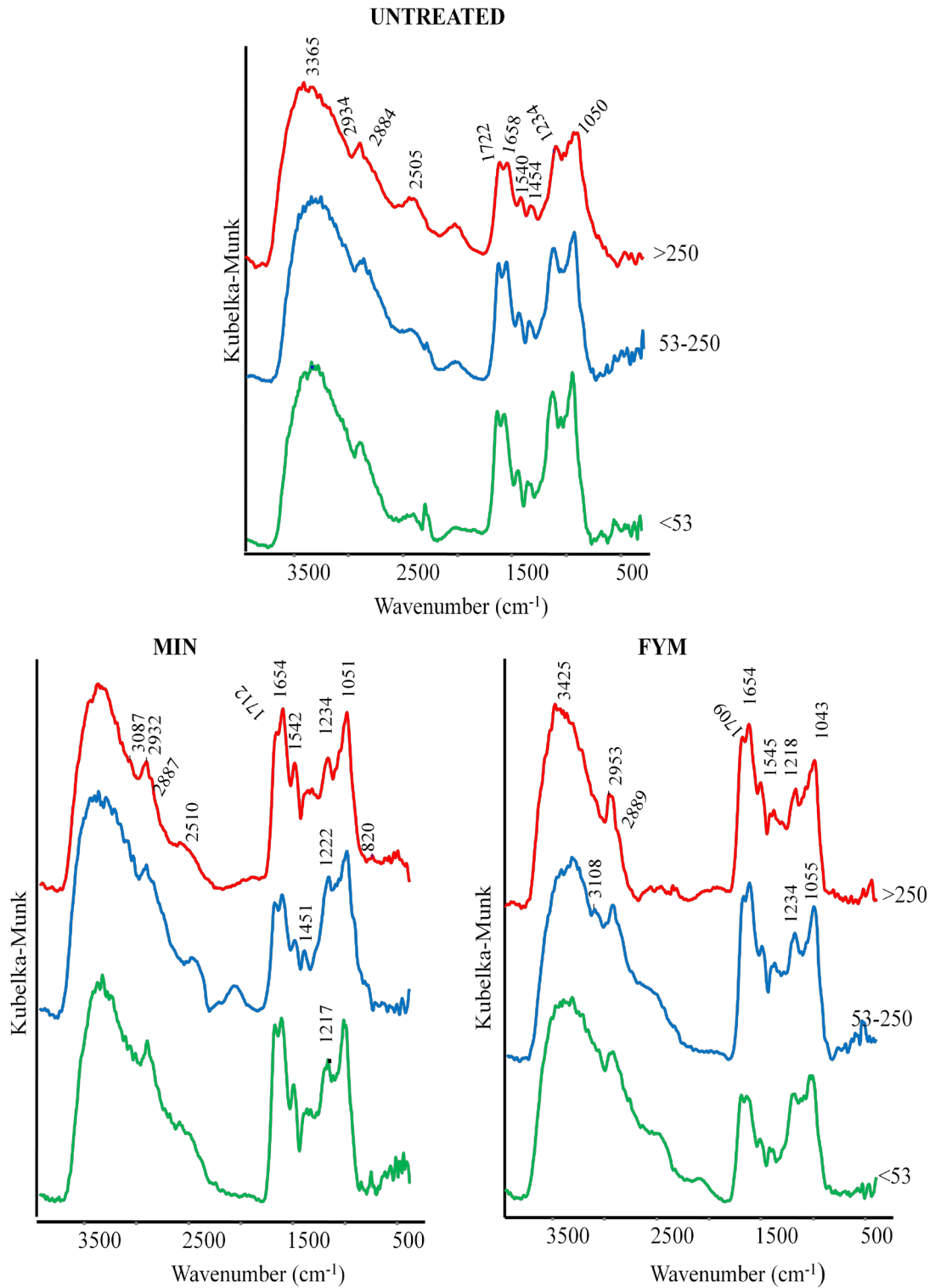
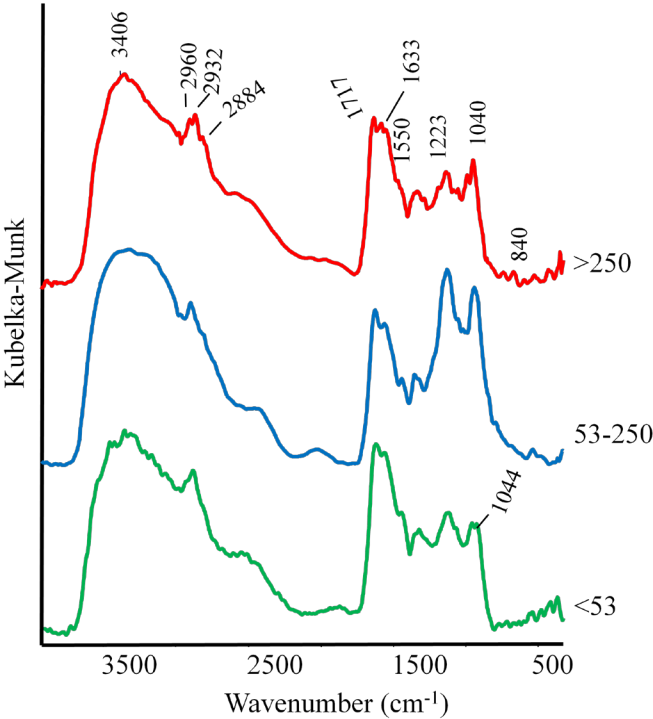


Figure 2b: Peaty soil; DRIFT spectroscopy spectra of HS from different aggregate size fractions

PEATY SOIL



MIN

FYM

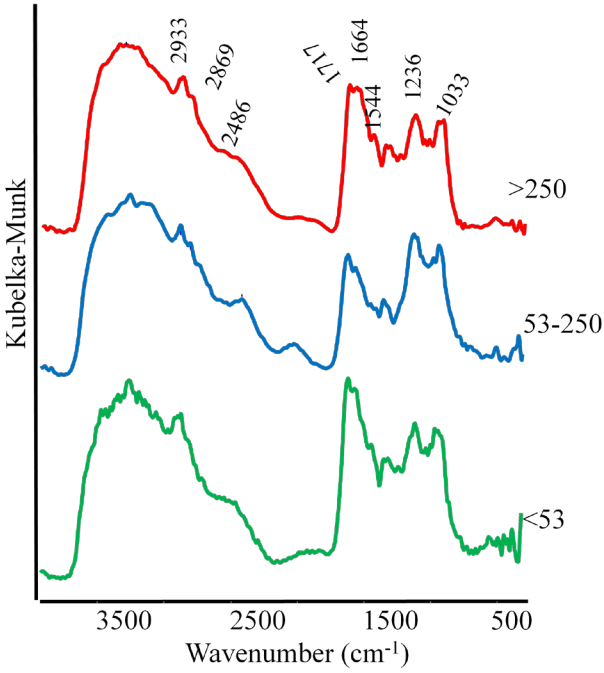
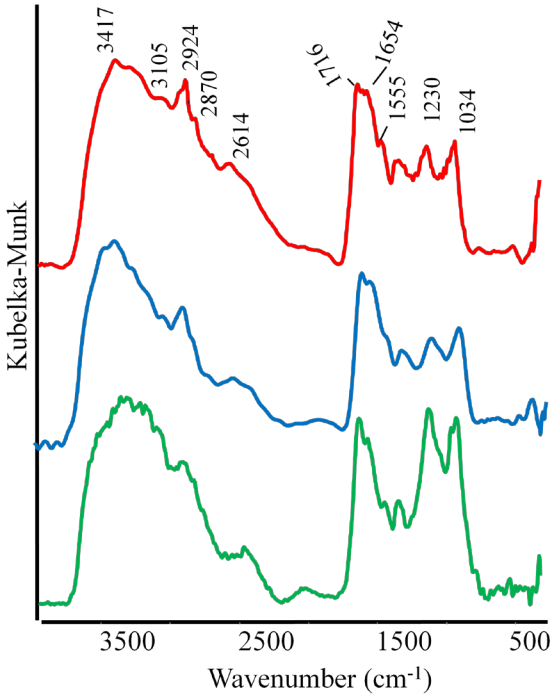


Figure 2c: Sandy soil; DRIFT spectroscopy spectra of HS from sand different aggregate size fractions

SANDY SOIL

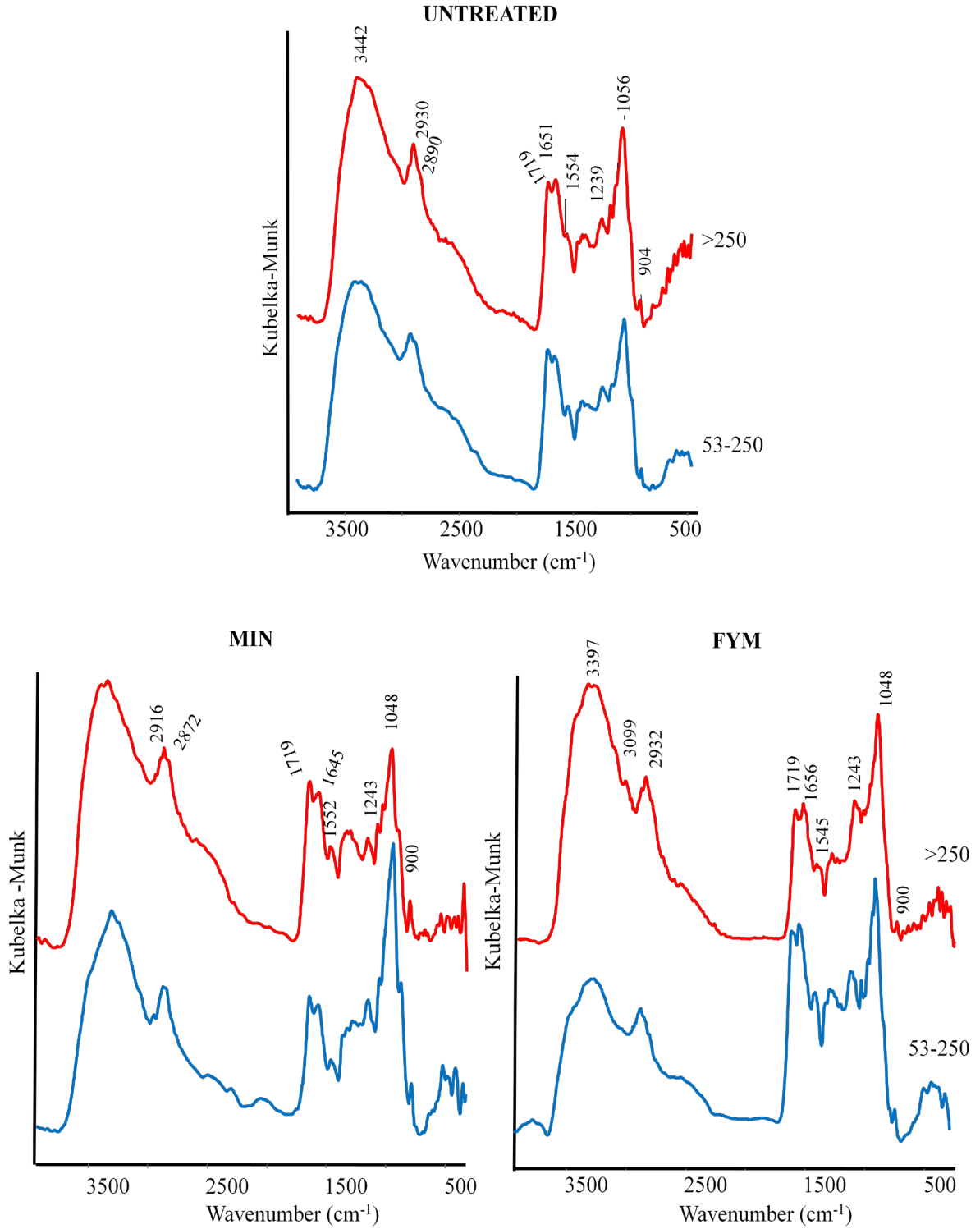


Table 4: Attributions of main functional groups of SOM extracted from different soil aggregates

Wavenumber (cm ⁻¹)	Functional Group	Possible Compound	Reference
4000–3200	O-H stretch	Alcohol, phenol, carboxylic acid	Rao, 1963
3100-3030	NH ₃ ⁺ stretch, C-H stretch	Peptide, Aromatic compounds	Rao, 1963
3000-2800	C-H stretch	Aliphatic chain	Rao, 1963
2700-2500	NH ₂ ⁺ stretch and OH stretch in dimeric acids	Peptide carboxylic acid	Rao, 1963
1705-1729	Carbonyl/carboxyl stretch	Peptide, Lignin	Rao, 1963, Boeriu et al., 2004
1650-1660	Amide I	Peptide	Rao, 1963
1540	Amide II	Peptide	Rao, 1963
1595, 1514	Aromatic skeletal vibration and CH deformation	Lignin	Boeriu et al., 2004, Kubo and Kadla, 2005
1460-1300	CH deformation and C-O stretch	Outer surface-suberin/cutin	Stewart, 1995
1375-1370	Phenolic OH region and aliphatic CH stretch	Lignin	Boeriu et al., 2004
1260-1240	C-O-C (ether), C-OCH ₃ in guaicyl group	Lignin	
1220-1215	C-C, C-O, C=O (combination)	Lignin	Boeriu et al., 2004
1160-1130	Glycosidic linkage (C-O-C)	Mixed polysaccharides	Kačuráková et al., 2000
1370, 1130-1050	Intense polysaccharide	Cellulose	Stewart, 1995
1000-800	Vibration of the pyranose ring	Glucose, galactose and mannose	Tul'chinsky et al., 1976

In FMY the relative intensities of carbonyl (1709 cm⁻¹) and C-O groups (1218 cm⁻¹), gradually increased from macro to silt clay fractions. A positive shift of the C-O group from macro to silt-clay fractions (16 cm⁻¹ for microaggregate and 22 cm⁻¹ for silt-clay fractions) might indicate an enrichment of C-O groups in phenolic structure, as also supported by NMR data. In general, HS structure of clay soil aggregate size-fractions seems to be influenced by both treatments. In particular the carboxyl groups were constantly more concentrated in silt-clay fractions, as also supported by NMR, while the C-O groups seem to be more sensitive to treatment type. Furthermore carbohydrates content (1110-1040 cm⁻¹) significantly decreased after the treatment with manure.

HS spectra of untreated peaty soil aggregate-size fractions are shown in Figure 2a. The main structural variations between aggregate size-fractions can be ascribed to

different peaks intensity of the aliphatic region (2930-2880 cm^{-1}), amide II (1550 cm^{-1}), C-O group (1223 cm^{-1}) and carbohydrates (1040 cm^{-1}). The aliphatic region was prevalently characterized by methylene group (2932 cm^{-1}) vibration whose intensity decreased from macro to silt-clay fractions. Only a weak shoulder due to methyl group (2960 cm^{-1}) stretching vibration appeared in macroaggregate size fraction. The gradual enhancement of amide II from macro to silt-clay might indicate preservation and accumulation of amino acid chains in the finest fraction. A considerable increase of C-O (1223 cm^{-1}) and carbohydrates groups (1040 cm^{-1}) characterized micro aggregate-size fraction, suggesting a preservation of sugar like substances, probably due to physical protection processes (Six et al., 2002).

In aggregates of mineral fertilised plots no substantial modification of functional groups between macro and microaggregates size fractions was observed. Conversely, the silt-clay fraction displayed an enhancement in relative intensities of the carbonyl group (1710 cm^{-1}), C-O group (1233 cm^{-1}) and carbohydrate (1048 cm^{-1}).

After 40 yrs of manure amendment there not were significant differences among functional groups attributable to manure. Substantially, the effect of treatments in peaty soil was not so strong to modify qualitatively the main functional groups into different aggregate size fractions, probably for the achievement of SOC saturation or close to saturation conditions (Chapter 1) in peaty.

In sandy soil the HS spectra (Figure 2c) were very similar between them and characterized by considerable presence of carbonyl groups (1719 cm^{-1}) and carbohydrates (1110-1040 cm^{-1}). An enrichment in carbohydrates might derive by partially undecomposed organic matter, rich in cellulose and hemicellulose (Turchenek and Oades, 1979; Jolivet et al., 2006) as a consequence of the a low biomass microbial activity in sandy soils. This may be interpreted as the occurrence of weak or no humification processes caused by a reduced microbial activities, hindered by sand low moisture contents. This is also supported by the lowest signal of carbohydrates in peaty and clay aggregate size fractions studied.

Conclusion

Long-term manure application caused a general enrichment in phenolic and aliphatic molecules in the humic fraction. Probably the lignin is the precursor of phenols. In soils, lignin is an important source for the formation of soil humic matter, an essential soil component that is known to have beneficial effects on soil physical, chemical and biological properties. Different stabilization processes, particularly related with aggregate size fraction. In silt-clay aggregates we evidenced especially spatial accessibility protection processes while selective preservation mechanisms were observed at microaggregate size fraction.

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**Chapter IV: Porosity in wet-sieved aggregates of different
soils and SOC protection processes**

Introduction

In the last decade increasing attention has been given to the physical separation of soil organic carbon (SOC), aiming to obtain homogeneous pools fractions which are related to different stabilization mechanisms. These are usually classified in chemical, physical and biogeochemical protection processes (Six et al., 2002; Lützow et al., 2006).

Many studies focused on aggregate stability and turnover (Six et al., 2002) mediated by many factors such as biota, ionic bridging, clay, carbonates and SOC (Bronick and Lal, 2005) that influence, in the end, the soil structure. Indeed, soil aggregate properties occur through complex interactions of physical processes, chemical associations, and biological activity (Oades and Waters, 1991, Tisdall, 1996, Jastrow and Miller, 1998 and Six et al., 2004).

A complex feedback involves the soil structure evolution and SOC turnover (Christensen, 2001), since the organic matter is a binding agent promoting aggregation, but is also protected by porosity. The soil pore distribution controls the spatial inaccessibility to microorganisms and the low O₂ diffusion, that are very important factors in SOC protection. Protection occurs because SOC availability to microorganisms is related with its position within the soil pore matrix and to micro-environmental conditions. The spatial distributions of SOC, solutes, and microbial communities within aggregates depend in fact on the pore development within aggregates (Chenu et al., 2001) and their interconnections that are accountable of the limited movement of organisms among different size categories of pores.

A simple hierarchical model for soil aggregation can explain many aspects of changes in soil organic matter degradation. Indeed, aggregate hierarchy occurs with a parallel hierarchy of pores that exist between and within aggregates of varying sizes (Elliott and Coleman, 1988). Thus, the smaller pores internal to microaggregates (53-250 µm) are more likely to exclude biota and their enzymes than those of larger structures (e.g. macroaggregates 250-2000 µm).

Porosity and micro structure within aggregates change with aggregate turnover (i.e., breakdown and reformation), altering activities associated with plant roots and soil fauna (Czarnes et al., 2000; Hussein and Adey, 1998), but also with tillage and

fertilizing activities that strongly affect aggregates stability. Schjøning et al. (2002) found a larger volume of pores $>30\ \mu\text{m}$ in an organically managed sandy-loam soil than in an arable one receiving only mineral fertilization. The former also evidenced as a much more complex and tortuous pore system. Pagliai et al. (2004) observed a change in shape and pore size distribution within about a year after compost and manure application. The amended treatments resulted in a higher macroporosity than the control, with the highest percentage of elongated pores (belonging to classes $50\text{--}500\ \mu\text{m}$) measured in the livestock manure treatment. On the contrary Haynes and Naidu (1998) found an increase in total porosity and the relative volume of pores $<30\ \mu\text{m}$ after 90 years of farmyard manure application. Although knowledge on the interaction between SOC and the aggregation process is rapidly increasing, the role of humic substances (HS) as binding agents, likely because of their heterogeneity and controversial origin, has been less studied and is still unclear, particularly the relation between aggregate porosity and the parameters that characterize HS quality and quantity.

Humic substances are operationally defined by a standardized procedure of extraction, but recent studies have shown that these are a heterogeneous pool of substances with very different turnover times (Lützow et al., 2007).

Thermal analysis, solid state nuclear magnetic resonance and pyrolysis have highlighted a heterogeneity of HA composition in the silt and clay fractions of the same soil and also among different soils (Mao et al., 2007). Kelleher and Simpson (2006), using advanced nuclear magnetic resonance approaches, suggested that the vast majority of operationally defined humic material is a very complex mixture of microbial and plant biopolymers and their degradation products, but not a distinct chemical category.

The aim of this study was to investigate the chemical and physical mechanisms of SOC protection in a long-term experiment established in the early 1960s in north-eastern Italy, where very different soil types fertilized were subjected to manure and mineral fertilization.

Material and Methods

2.1 The long-term experiment

The long-term trial is located at the Experimental Farm of the University of Padova (Veneto Region, NE Italy). The local climate is sub-humid, with annual rainfall of about 850 mm and yearly average temperature of 12 °C. The reference evapotranspiration (ET_o) is 945 mm with a peak in July (5 mm d⁻¹). ET_o exceeds rainfall from April to September. The site has a shallow water table ranging from about 0.5-1.5 m in late winter-early spring to 1-2 m in summer.

This experiment began in 1964 in 4 m² open lysimeters, 80 cm deep. The experimental treatments derive from the factorial combination of three types of soil (hereinafter called clay, sand and peaty in relation to their dominant property in Table 1) with six types of mineral, organic or mixed fertilization, organized in two randomized blocks (36 lysimeters).

The soils were brought from three locations in the Veneto region: clay soil from the south-western plain, sandy soil from the central coastal area and peaty soil from the southern plain. The original soil profiles were reconstructed in the lysimeters. The sandy soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) contain predominantly quartz and feldspar and a significant amount of dolomite (16 %). Clay soils (Cumulic, Vertic, Endoaquoll fine, mixed, calcareous, mesic – ARPAV, 2005) have the higher amount of smectite / montmorillonite (16 %) than the other soils and a considerable presence of mica (19%) and dolomite (15 %). The peaty soils (Typic Sulfisaprists euic, mesic – ARPAV, 2005) are characterized by a higher content of mica (25 %) whereas smectite / montmorillonite reach the 12 %. After 40 years of experimental conditions, some original proprieties are changed as reported recently in Morari et al., (2008) and in table 1.

Table 1: Physical-chemical characteristics of the 0-30 cm depth at the beginning of the experiment (1964).

Parameters	clay	sandy	peaty
Sand (2 mm –50 µm) (%)	25.0	93.4	38.0
Silt (50-2 µm) (%)	23.0	6.0	13.6
Clay (<2 µm) (%)	52.0	0.6	48.4
pH	7.9 (7.7)	8.1 (7.7)	4.9 (7.2)
Total Carbonate (g kg ⁻¹)	26.0	39.0	0.0
Organic Carbon (g kg ⁻¹)	14.5 (15.7)	1.7 (3.1)	105.0 (89)
Total Nitrogen (g kg ⁻¹)	1.5 (1.5)	0.15	6.7 (6.5)
C/N	10.0 (10.2)	12.0	16.0 (13.6)
Total Phosphorus (g kg ⁻¹)	2.8 (5.6)	0.5 (1.8)	1.1 (3.1)
Available Phosphorus (mg kg ⁻¹)	161.5	26.2	100.4

Values between brackets were measured in 2002

Fertilization treatments considered for this study were as follows: no applications (O); farmyard manure – F2 (40 t ha⁻¹ yr⁻¹); mineral fertilizer – M2 (200 kg ha⁻¹ yr⁻¹ N - 100 P₂O₅ - 240 K₂O). The F2 applied fairly the same amount of macroelements than M2 and about 3.5-4 t C ha⁻¹ y⁻¹. Until 1984 there was a two-year maize (*Zea mays* L.) - wheat rotation (*Triticum aestivum* L.). Thereafter, a variable rotation was adopted between 1985 and 1992, with various horticultural crops. Since 1993 there has been a three-year rotation of tomato (*Lycopersicon esculentum* Mill.) – sugarbeet (*Beta vulgaris* L.) – maize, followed by various horticultural crops, maize and sunflower (*Helianthus annuus* L.) from 2003 to 2007. Apart from fertilization, all plots were treated in the same way in terms of rotation and management (tillage, sowing, harvest, etc). The top 15-20 cm was dug each autumn and crop residues were removed from all treatments.

2.2 Soil sampling and fractionation technique

The top soil layer (0-20 cm) was sampled in June 2008. The samples were taken from five different points in the whole lysimeter area and bulked to obtain a sample of about 1 kg. Samples were broken up manually, air-dried and sieved at 2 mm. To standardize the fractionation procedure and better evidence the relationship between aggregation and organic binding agent in the different soils, we first isolated the large macroaggregates (from 1 to 2 mm) by dry-sieving. More than 50-60% of the total mass was accounted in the 1-2 mm aggregate fraction. No large aggregates were isolated from sandy soil, so all the soil was retained. Large aggregates (two sub-samples per plot - 36 in total) were then separated into three aggregate size fractions by wet-sieving, using an automatic machine oscillator. Briefly, the apparatus produces vertical actions which transmit a 3 cm stroke vertical movement to a stack of interfitting sieves with a mesh of 250 and 53 μm . The sieves, 10 cm in diameter and 5 cm height, were immersed in distilled water inside a beaker (15 cm diameter x 18 cm height). Prior to sieving, the water level was adjusted to prevent water reflux over the edge of the 250 μm sieve during the oscillation.

Before sieving, 15 g of sample were immersed on top of the 250 μm sieve for 10 min to allow slaking. After slaking, aggregates were separated by vertical oscillations at a frequency of 50 rpm for 18 min (for a total of 900 oscillations). Macroaggregates (2000-250 μm) and microaggregates (53-250 μm) were then collected from the corresponding sieves, whereas the silt-clay fraction (<53 μm) remaining in the beaker was precipitated by adding 3 ml of 1N CaCl_2 . In sandy soil the silt-clay fraction was not recovered due to its very low amount and in general, due to the lack of aggregation, we can define macroaggregates (2000-250 μm) and microaggregates (53-250 μm) as coarse and fine sand respectively. All isolated fractions were dried at 60 $^\circ\text{C}$ in a forced-air oven.

2.3 MIP (Mercury Intrusion Porosimetry)

Mercury intrusion porosimetry (MIP) is commonly accepted as a standard of total pore volume and pore size distribution determination. The different aggregates fractions and the starting bulk macroaggregates (1000-2000 μm) were analyzed by MIP for analyzing the total porosity (TP) and the pore size distribution (PSD), within

the range of 0.0035–75 μm . Pores within the range 10–75 μm were analyzed with Thermo Finnigan Pascal 140, while pores within the range 0.0035–10 μm were analyzed with Thermo Finnigan Pascal 240. The pore radius into which Hg was intruded was calculated as a function of pressure using the Washburn equation:

$$Pr = -2\lambda \cos(\theta)$$

where P is pressure (kPa), r is radius (μm), λ is surface tension of mercury (0.47 N m^{-1}), and θ is contact angle (140°). Pores were classified according to Cameron and Buchan (2006) in the following ranges: meso-pores 30–75 μm (C30–75), micro-pores 5–30 μm (C5–30), ultramicro-pores 0.1–5 μm (C0.1–5), crypto-pores 0.01–0.1 μm (1) (C0.01–0.1), crypto-pores (2) 0.007–0.01 μm (C0.007–0.01) and crypto-pores (3) 0.0035–0.007 μm (C0.0035–0.007)

2.4 Humic carbon extraction

The humic carbon of the different aggregate fraction (WSA) were extracted from the air-dried samples with 0.5 M NaOH (1:10 w/v) in Dubnoff bath at 50 °C for 16 h and were separated from the suspended material by centrifuging at 15000 rpm for 15 min. Here, the term humic substances is the fraction soluble in bases and comprehensive of humic and fulvic acids. Humic extracts (50 ml) were transferred into 18,000 mol. wt cut-off dialysis Visking tubing (Medicell Ltd., London, UK) and dialyzed against double-distilled water. The water was changed daily until the liquid outside the dialysis tube was colorless. Moreover, the samples were treated with an ion exchange on Amberlite IR 120 H^+ (Stevenson, 1994) to remove other cations. Finally the humic extracts were dried by lyophilizing before spectroscopic analyses. Prior to perform chemical analysis HS extracted from the two replicates were mixed together to form a single sample. In total 24 HS samples were analyzed (3 soils X 3 treatments X 3 aggregate fractions minus 3 silt-clay fractions in sandy soil).

2.5 Gel-permeation chromatography

Gel-permeation chromatography of humic extract was conducted on a Sephadex G-100 gel packed in a 70 x 1.6 cm Pharmacia column (Pharmacia, Uppsala, Sweden). The gel packing solution and eluent were 0.02 M Na₂B₄O₇. The apparent molecular weight of the fractions was: above 60 kDa (HF1); between 60 and 30 kDa (HF2) and less than 30 kDa (HF3). Column calibration was based on previously assessed standard proteins (Kit MS-II, Serva, Heidelberg, Germany) (Martin et al., 2006). Humic fraction analysis was done for each soil fraction, except for the not recovered <53 µm one of sandy soil.

2.6 Solid state ¹³C-nmr

Solid state ¹³C NMR characterization was performed on a Bruker Avance 400 WB spectrometer equipped for solid state analysis and operating at 100.61 MHz. Samples were spun at 12.5 kHz in 4 mm diameter zirconia rotors with Kel-F caps. The ¹³C SPE (SPE=single pulse experiment) MAS NMR spectra were obtained with high power proton decoupling during acquisition, 30 seconds relaxing delay (D1), 3072 scans (NS) , and processed with a 100 Hz exponential line broadening. ¹³C chemical shifts were externally referenced to solid sodium 3-(trimethyl-silyl)-1-propane sulfonate at 0 ppm. Magic angle conditions were adjusted by observing ⁷⁹Br spinning side bands pattern in a rotor containing 5% of KBr.

It is noteworthy that collected spectra reveal the presence of a several kHz wide undesired band, which is superimposed to the resonances assigned to humic substances. That wide band is due to the presence of carbon atom inside the probe and rotor cap materials. For this reason, the ¹³C SPE MAS NMR spectrum of an empty rotor was recorded by employing the same acquisition parameters above described and then subtracted from each measurement collected in presence of sample: the obtained ¹³C SPE MAS NMR spectra were employed for quantitative analysis of humic substances. All molecular moieties detectable in samples were grouped into five main chemical functionality classes. Each class was quantified by integrating resonance signals about the following ppm ranges: 182-158 (carbonyl C), 158-108 (aryl-C), 108-90 (anomeric-C), 90-35 (O, N-alkyl C), 35-5 (alkyl-C).

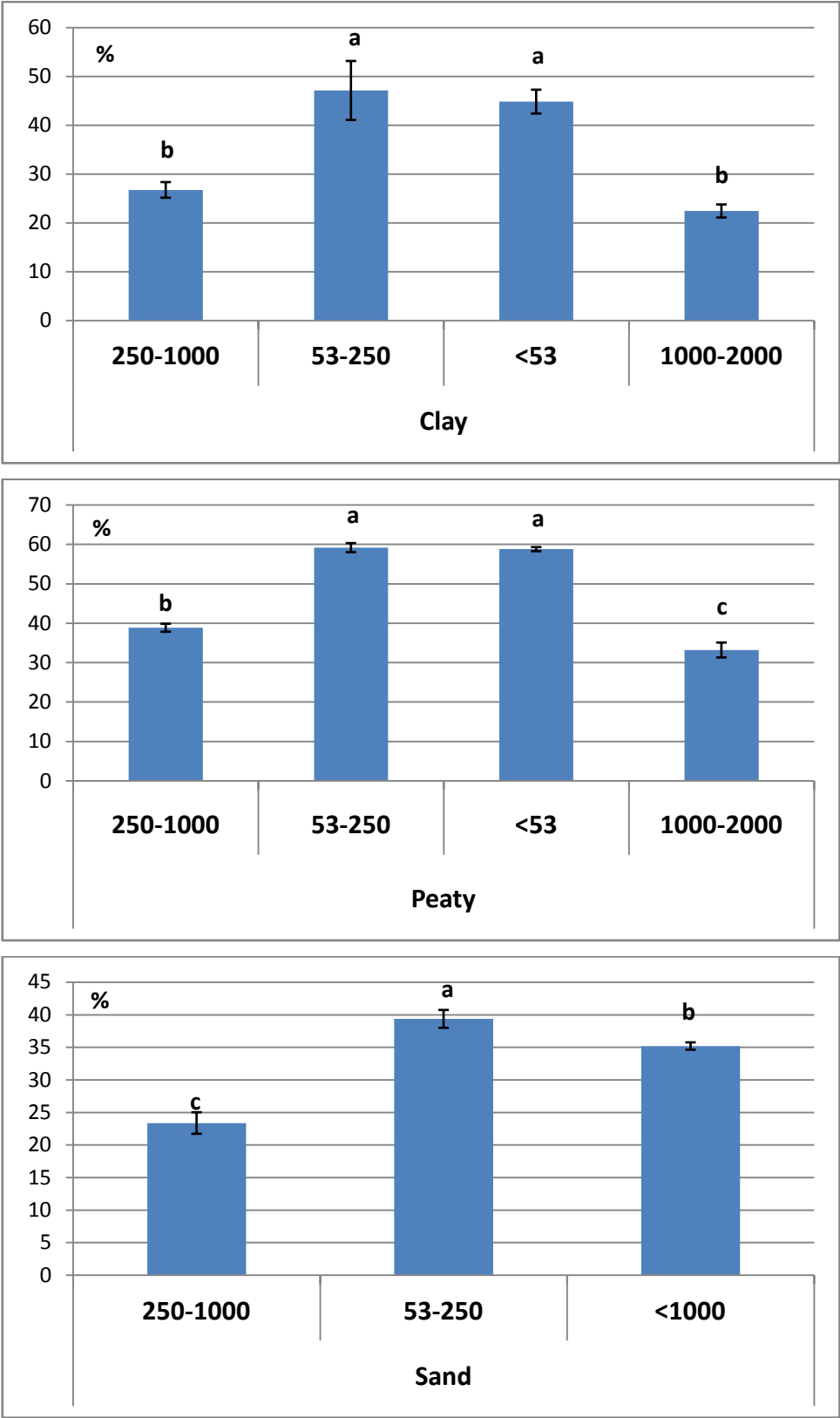
2.7 Statistical analysis

To ¹³C NMR characterizations HS extracted from the two replicates were mixed together to form a single sample. In total 24 HS samples were analyzed (3 soils X 3 treatments X 3 aggregate fractions minus 3 silt-clay fractions in sandy soil). Replicates on HS were not performed for the long measurement times. Soil porosity data were analyzed with two-way ANOVA and significantly different means were differentiated with the Student–Newman Keuls test (StatSoft Inc., 2004). To clarify the structure of these interdependences, we performed a joint principal component analysis (PCA) of our data on the 15 variables: the six porosity classes (relative values), the total porosity %, HC, HC/OC, three HFs expressed in qualitative (HF2-r and HF3-r) and, and the five chemical functionality classes given by NMR analysis. Variables were standardized and submitted to the PC analysis; rotated orthogonal components (varimax method of rotation) were extracted and the relative scores were determined. Only PCc with eigenvalues > 1 were considered for the discussion.

Results

Porosity varied according to aggregate fraction while no differences were evidenced between the treatments. Total Porosity (TP) of the aggregate fractions is shown in figure 1. In each soil the higher TP was measured in the microaggregates (53-250 µm) and silty-clay (<53 µm) fractions (missing in sandy soil). The highest values were observed in the peaty soil ($p < 0.01$) with a TP of 59% in both the two fine fractions and 39% and 33% for the macroaggregates (2000-250 µm) and bulk fraction (1000-2000 µm), respectively. In a similar way clay soil had a higher TP in microaggregates (47%) and in silt clay fraction (44%) than macroaggregates and bulk fraction. Low TP was measured in the sandy soil with 39% in fine sand (53-250 µm) and 23% in coarse sand particles (2000-250 µm). For the bulk sand a TP of 35% was measured.

Figure 1: Total porosity for the three soils in the different aggregate fractions



In clay soil, pore size distribution (PSD) changed significantly ($p < 0.01$) according to the aggregate fractions (figure 2a-b) but not treatments even if in porosity classes $< 5 \mu\text{m}$ an apparent increase of volume associated with farmyard manure application was observed. Higher meso-pore volumes were observed in the macro and microaggregates fractions (0.07 and $0.09 \text{cm}^3 \text{cm}^{-3}$) than silt clay. On the contrary micro-pores domain exhibited higher volumes in microaggregates ($0.30 \text{cm}^3 \text{cm}^{-3}$) than silt clay and macroaggregates ($0.07 \text{cm}^3 \text{cm}^{-3}$). Ultra micro-pores were higher in silt clay fraction ($0.20 \text{cm}^3 \text{cm}^{-3}$) than the other two classes while crypto-pores (1) to crypto-pores (3) showed similar distributions with higher values in macroaggregates.

In peaty soil, PSD changed significantly between the aggregate fractions (figure 2a-b). As well as observed for clay, treatments didn't affect the porosity even if an increase of volumes in classes $<$ crypto-pores seemed to be associated with farmyard manure.

Meso-pores and micro-pores were higher in microaggregates (0.12 and $0.31 \text{cm}^3 \text{cm}^{-3}$) than macroaggregates and silt clay fraction while ultra micro-pores was the most frequent class in silt clay fraction ($0.38 \text{cm}^3 \text{cm}^{-3}$, corresponding to the 60% of the TP). From the crypto-pores to the lower classes the distribution was similar to the clay soil with higher values in macroaggregates ($p < 0.01$) than microaggregates and silt clay fractions. The latter didn't differ statistically and showed values very close to zero in the crypto-pores (2) and crypto-pores (3).

The only treatment effect ($p < 0.01$) was observed in meso-pore class of sandy soil, with higher values in mineral treatment ($0.20 \text{cm}^3 \text{cm}^{-3}$) than FMY ($0.13 \text{cm}^3 \text{cm}^{-3}$) and control ($0.15 \text{cm}^3 \text{cm}^{-3}$). In the same soil, mesopores, micropores and cryptopores were more frequent in fine sand ($53\text{-}250 \mu\text{m}$) than coarse one ($250\text{-}1000 \mu\text{m}$) (figure 2a-b). The other classes (ultramicro-pores, crypto-pores (2)(3)) did not exhibit a significant difference between the fractions.

Figure 2a: Distribution of soil total ($\text{cm}^3 \text{cm}^{-3}$) porosity in the range 0.01–75 μm for the different soils considered. Within each porosity class, histograms having the same letter are not statistically different at $P < 0.05$. Bars represent the standard error.

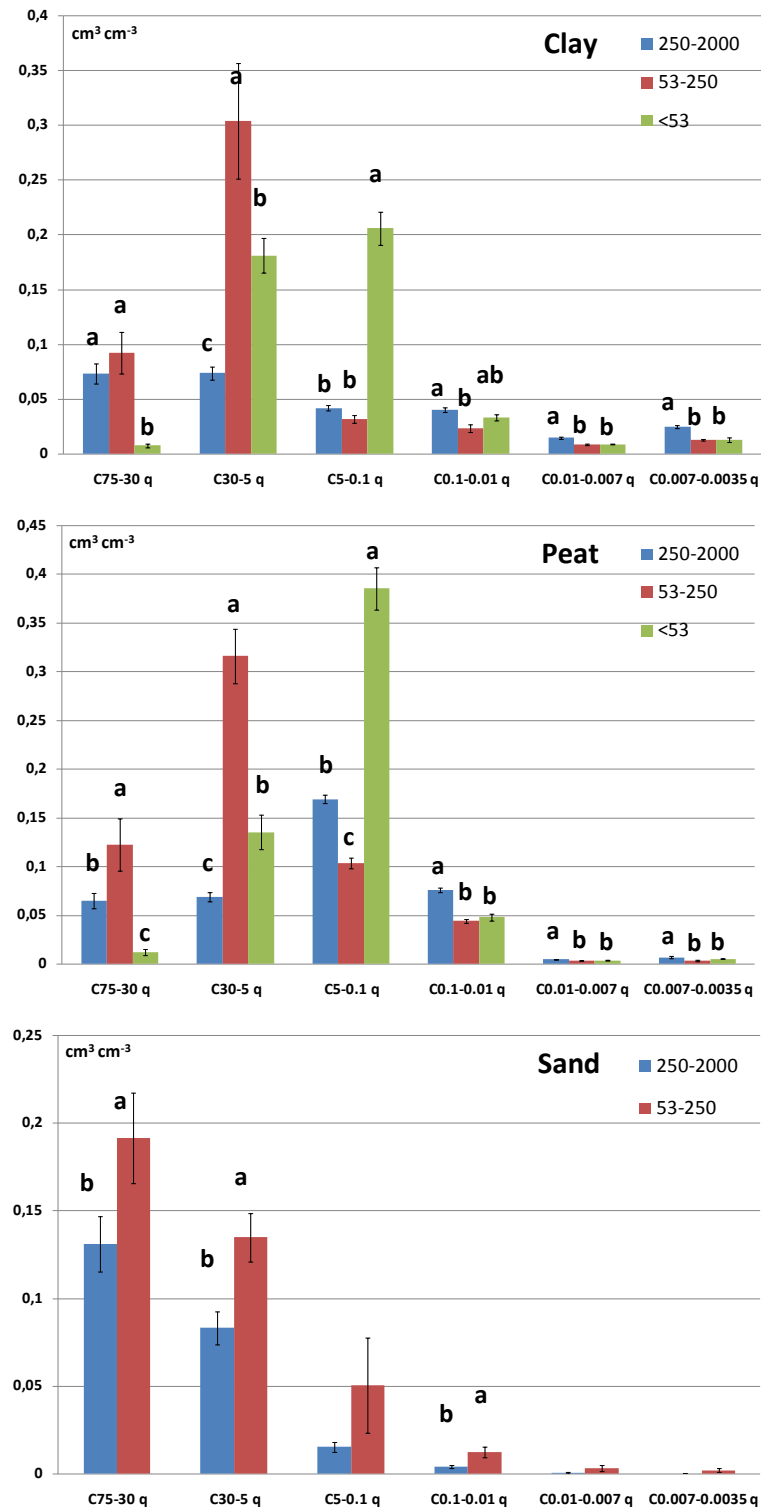
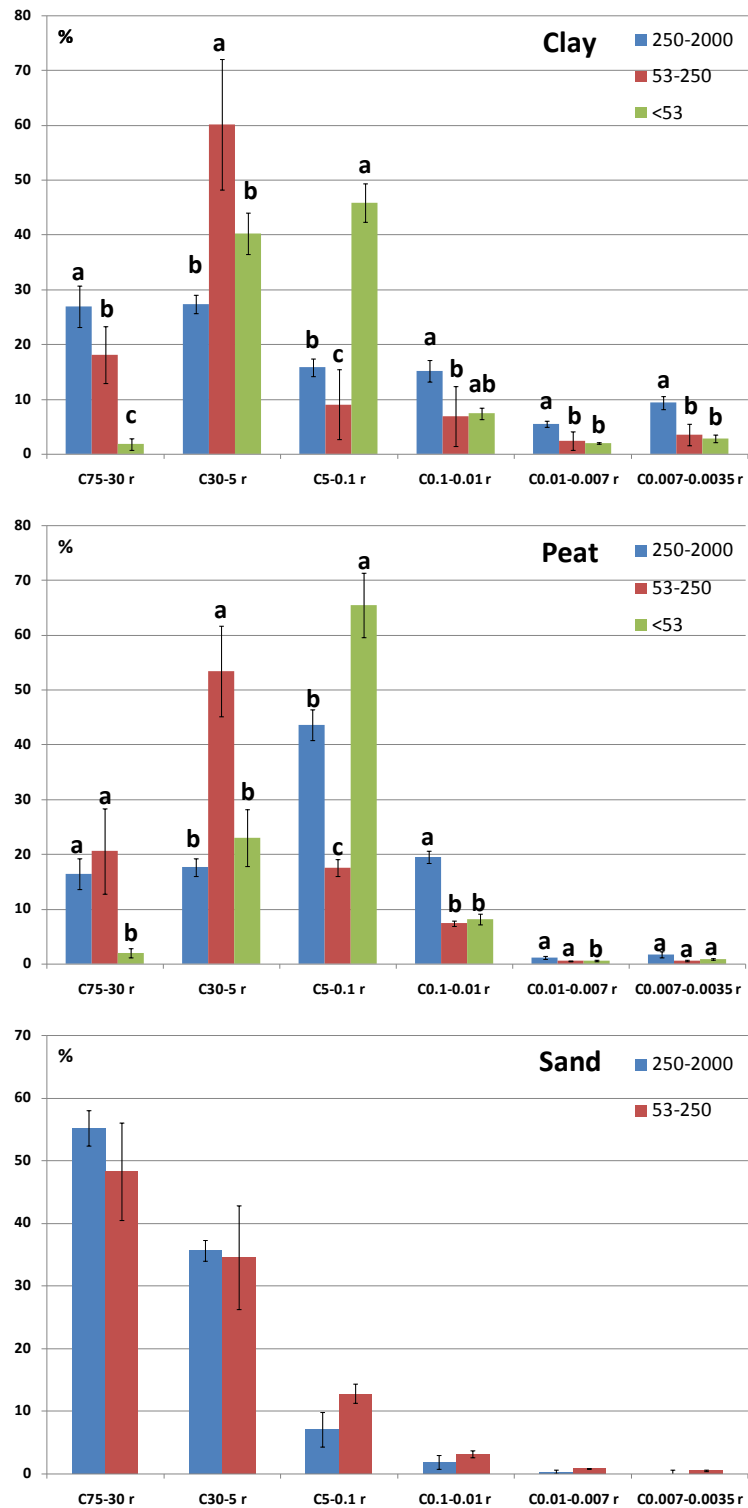


Figure 2b: Distribution of soil total relative (%) porosity in the range 0.01–75 μm for the different soils considered. Within each porosity class, histograms having the same letter are not statistically different at $P < 0.05$. Bars represent the standard error.



Details on NMR analysis and results are reported in Chapter 2. The correlation matrix (Table 1) evidenced significant values between pore classes, (expressed either as relative as well as quantitative way), OC and HC, HS fractions (HF1, HF2 and HF3) and the functional groups. The meso-pores (relative) were positively correlated ($p < 0.05$) with O, N-alkyl C ($r = 0.64$) and anomeric C ($r = 0.80$) and negatively with alkyl-C ($r = -0.64$) and carbonyl-C ($r = -0.86$). Ultramicro-pores and crypto-pores (1) had positive correlation with aryl-C ($r = 0.46$ and $r = 0.42$) and carbonyl-C ($r = 0.60$ and $r = 0.40$) and a negative correlation with O, N-alkyl C ($r = -0.59$ and $r = -0.51$) and anomeric-C ($r = -0.53$ and $r = -0.57$). C0.01–0.1 q evidence a significant correlation with aryl-C ($r = 0.70$), carbonyl-C ($r = 0.52$) and alkyl-C ($r = 0.45$) compounds. Crypto-pores (1) (quantitative) had positive correlation with alkyl-C ($r = 0.48$)

Application of PCA to soil data allowed four factors to be extracted, explaining the 89% of the variability (Table 2). The first factor explained 45% of the variance and was correlated (factor loadings > 0.7) with the majority of the OC qualitative factors (CU, HF2-r, HF3-r), and the aryl-C functional group. The second factor explained 20% of the variance and was correlated with crypto-pores (2) and crypto-pores (3). The third factor explained 14% and was correlated with two porosity classes (meso-pores and ultramicro-pores) and the carbonyl C - anomeric C functional groups. The fourth factor explained 9% of the variance and was correlated with micro-pores. The plots of the variables and cases according the first three factors are reported in figure 2a and 2b. Factors 1 and 2 (figure 2a) allowed to identify two clusters represented by the peaty and clay macroaggregates which were mainly isolated by aryl-C, CU and alkyl-C, alkyl-C and crypto-pores (1)(2)(3) respectively. Moreover the plots according to factors 1 and 3 (figure 2b) allowed to identify two clusters, the silty-clay fraction of clay and fine and coarse sand of sandy soils, which were mainly isolated by carbonyl C and crypto-pores (1) and O, N-alkyl C, anomeric C, and meso-pores, respectively.

Table 1

Correlation matrix of selected soil parameters (value in bold are significant different at P = 0.05).

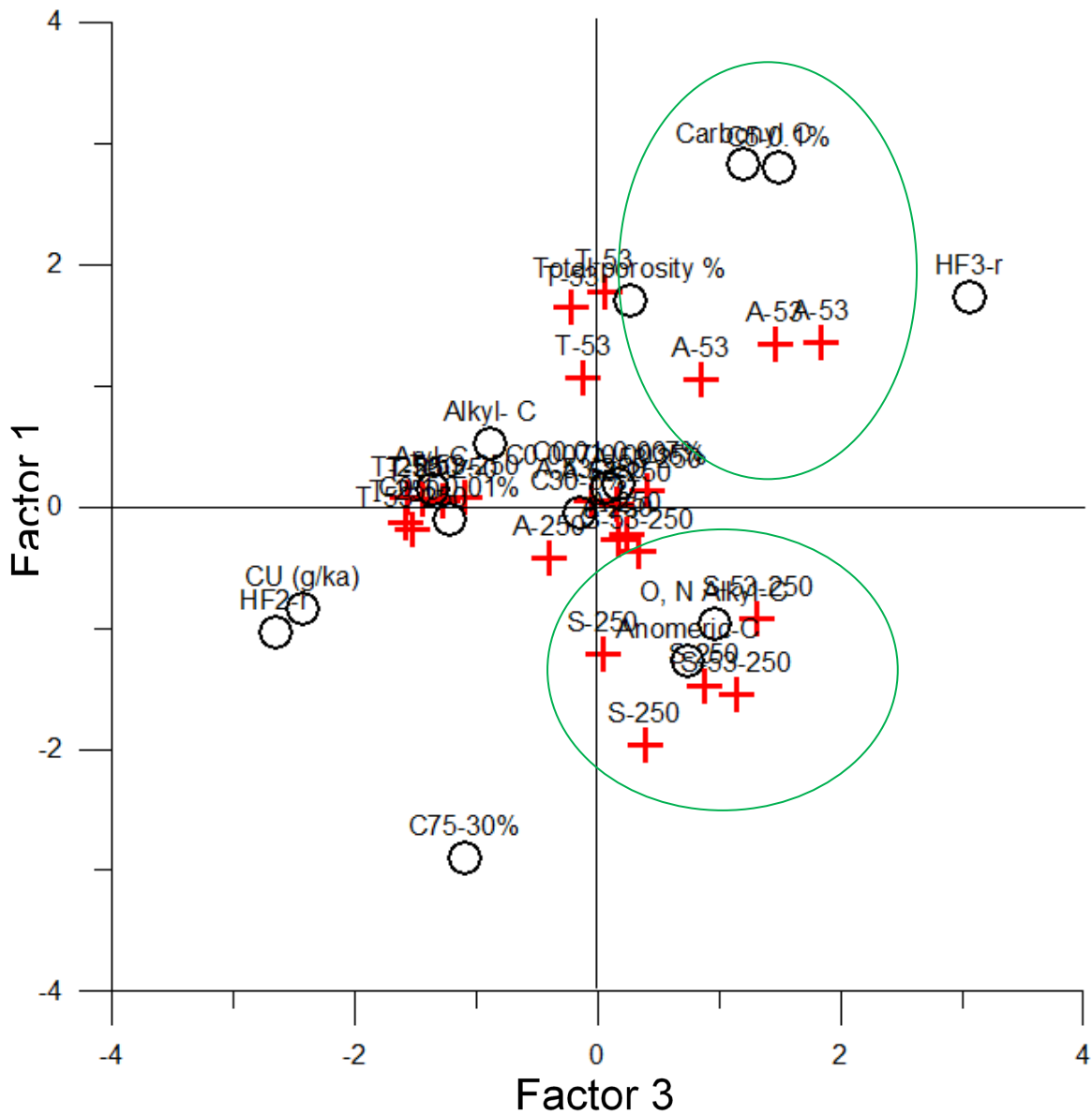
	OC (g/ka)	CU (g/ka)	HF1%	HF2%	HF3%	Carbonyl C	Aryl-C	Anomeric-C	O, N Alkyl-C	Alkyl- C
C75-30%	-0,44	-0,37	0,35	-0,34	0,18	-0,86	-0,34	0,80	0,64	-0,64
C30-5%	-0,13	-0,12	-0,11	-0,22	0,21	0,05	-0,22	-0,02	0,11	0,20
C5-0.1%	0,39	0,32	-0,29	0,30	-0,16	0,60	0,46	-0,53	-0,59	0,33
C0.1-0.01%	0,59	0,58	0,07	0,61	-0,53	0,40	0,42	-0,57	-0,51	0,36
C0.01-0.007%	-0,17	-0,19	0,01	0,01	-0,01	0,33	-0,35	-0,23	0,17	0,17
C0.007-0.0035%	-0,13	-0,15	0,02	0,06	-0,06	0,33	-0,32	-0,25	0,15	0,18
C75-30Q	-0,22	-0,15	0,27	-0,22	0,10	-0,75	-0,13	0,71	0,40	-0,54
C30-5Q	0,18	0,16	-0,14	0,03	0,02	0,23	0,09	-0,23	-0,23	0,35
C5-0.1Q	0,38	0,31	-0,33	0,30	-0,15	0,61	0,49	-0,51	-0,62	0,29
C0.1-0.01Q	0,83	0,80	-0,04	0,72	-0,59	0,50	0,70	-0,69	-0,80	0,48
C0.01-0.007Q	-0,15	-0,19	-0,14	-0,02	0,06	0,49	-0,34	-0,33	0,09	0,30
C0.007-0.0035Q	-0,13	-0,16	-0,10	0,05	-0,01	0,48	-0,33	-0,34	0,09	0,28

Table 2:

Factor loadings (varimax normalized) calculated for selected soil parameters.

	Factor 1	Factor 2	Factor 3	Factor 4
C75-30%	0,182764	0,142530	<u>-0,949080</u>	0,081910
C30-5%	0,174812	0,156181	-0,003952	<u>-0,936466</u>
C5-0.1%	-0,141554	0,140998	<u>0,814080</u>	0,473846
C0.1-0.01%	-0,584986	-0,559108	0,268290	0,401501
C0.01-0.007%	0,072179	<u>-0,962184</u>	0,060957	0,037349
C0.007-0.0035%	0,033570	<u>-0,957909</u>	0,072674	0,039138
Total porosity %	-0,225402	0,518364	0,631348	-0,371972
CU (g/ka)	<u>-0,886532</u>	0,175788	0,226720	0,034407
HF2-r	<u>-0,934960</u>	-0,054747	0,206006	0,052205
HF3-r	<u>0,940680</u>	0,083850	-0,016309	-0,022848
Carbonyl C	-0,136843	-0,172626	<u>0,893980</u>	-0,004166
Aryl-C	<u>-0,723718</u>	0,426569	0,382282	0,248727
Anomeric-C	0,582347	0,223775	<u>-0,713539</u>	0,161107
O, N Alkyl-C	0,691662	-0,250466	-0,628364	-0,087583
Alkyl- C	-0,378462	-0,315760	0,431119	-0,476388
% Total variance	45,22	20,03	14,11	9,94

Figure 2b: PCA plots of the variables and cases ; factors 1 and 3.



Discussions

Soil porosity distribution strongly affects the SOC dynamic, in some cases promoting a faster SOC turnover but in others its protection/stabilization by spatial inaccessibility against microorganisms and enzymes. This occurs in the micro to ultra micro-crypto porosity domain, where organic matter could interact as well with clay minerals by intercalation or adsorption (Lützwow et al., 2006). Strong et al. 2004

studying the spatial location of C composition in the soil pore system, found a strong correlation ($r = 0.92$) between total C and pore volume $<1.2 \mu\text{m}$. Conversely they observed a negative correlation ($r = -0.35$) for total C and pore volume $15\text{--}60 \mu\text{m}$. These results are consistent with our data, since correlation between OC and crypto-pores (C0.1–5) and meso-pores (C30–75) was 0.83 and -0.24, respectively. The SOC protection from decomposition is enhanced in small microaggregates that are rich in pore $<0.2 \mu\text{m}$ diameter, which is considered to be the limiting size for access by bacteria (Cameron and Buchan, 2006; Lützow et al., 2006). Pagliai and Nobili (1993) showed that soil enzymatic activity was generally related to the size of the soil microbial population and that it was positively influenced by the number of pores, ranging from 30 to 200 μm .

Moreover Kaiser and Guggenberger (2003) suggested sorption of SOC at the mouth of very small micropores ($<2 \text{ nm}$) and their clogging, with strong bonding that could give protection against chemical and biological attack.

Humic substances, generally considered recalcitrant, are formed by microbially mediated composition of biological materials into humic monomers (HF3) and abiotic polymerization of humic monomers to form humic substances with different degrees of polycondensation (HF2, HF1,) (Stevenson, 1994).

Although this “traditional” theory is still in discussion (Piccolo, 2002; Lützow et al., 2006), numerous laboratory studies demonstrated that clay increased polymerization and stabilization of humic substances by adsorption of humus monomers (HF3) on clay surfaces (Laird et al., 2001). In our study we can't report a significant relationship between HF fraction and porosity classes, but for cryptopores and HF3 ($r=-0.59$) and HF2 (0.73).

Conversely, NMR analysis allowed to identify significant correlations between functional groups and porosity. In particular, a strong correlations between meso-pores (C30–75 q) and O, N-alkyl C ($r=0.45$) and anomeric C ($r=0.73$) were observed.

They suggest that larger pores are associated with immature HS composition characterised by preserving features very close to that of the plant residues, in particular related to amino acids and carbohydrates substances. This finding is

supported also by the relative abundance of meso-pores in the macro and micro aggregate fraction. In particular macroaggregates have been found stabilized by transient organic materials such as microbial- and plant-derived polysaccharides or temporary binding agents such as fungal hyphae and roots (Six et al., 2004).

Micropores, abundant in microaggregates and silt clay fraction, evidenced a weak correlation (0.34; $P = 0.07$) with alkyl-C compounds represented by fats and waxes deriving from plant, fungi and bacteria. Alkyl-C and carbonyl-C compounds can interact with mineral surfaces, i.e. ligand exchange, polyvalent cation bridges, and weak interactions, such as hydrophobic interactions including van der Waals forces and H-bonding (Vermeer & Koopal, 1998; Vermeer et al., 1998). Chenu & Stotzky (2002) suggested that small molecules sorbed to mineral surfaces cannot be utilized by microorganisms unless they are desorbed so that they can be transported into the cell. The adsorption of macromolecules is considered non-reversible (Chenu & Stotzky, 2002) and associated with conformational changes that render macromolecules unavailable to the action of extracellular enzymes (Theng, 1979; Khanna et al., 1998). Alkyl-C is considered a particularly recalcitrant form of soil C (Derenne & Largeau, 2001; Baldock et al., 2004). The recalcitrance of alkyl-C compounds is evident from the selective preservation of such compounds during biodegradation of soil OM, as reviewed by Baldock et al. (1997). Hu et al. (2000) proposed that this resistance can be partly due to the semicrystalline nature of the polymethylene, which may derive from aliphatic biopolymers.

Silty-clay fractions (<53 μm) exhibit the higher amount of ultramicro-pores (C0.1–5) that are correlated with carbonyl-C and aryl-C. Aryl-C compounds and polymers are reported as the most resistant to degradation. Their precursors are compounds such as in lignin and a range of polymethylenic molecules, such as lipids and waxes, cutin and suberin (Derenne & Largeau, 2001).

Cryptopores (1), which evidenced a positive correlations with aryl-C and carbonyl-C, were found in higher amounts in macroaggregates. Most likely <0.01 μm porosity was influenced by the internal porosity of OM, represented by coarse OM acting as temporary binding agents in the macroaggregates (Tisdall and

Oades 1982). This is also supported by the observations in sand soil that reveal higher crypto-pores (1) in the fine sand than in the coarse in a complete lack of aggregation.

In general, the spatial inaccessibility principle seems to be the principal protection process that involve the HS (interaction and intercalation with/in the mineral surfaces), in particular associated with silt clay fraction and microaggregates.

This is confirmed by PCA plots: a) in sand soil, the fine and the medium sand tend to be differentiated by labile HS compounds (O, N-alkyl C and anomeric C). This may be interpreted as the occurrence of weak or no humification processes caused by a reduced microbial activities, hindered by sand low moisture contents; b) Aryl-C compounds tend to be associated with micro and macro aggregates in peaty soil, principally associated with meso and micro-pore classes. These compounds are generally considered recalcitrant and for this reason they accumulated in the bigger pore classes, evidencing selective preservation processes; c) Alkyl-C compounds are associated with crypto-pores (1)(2)(3) in macro and microaggregates of clay soil while carbonyl-C tend to be associated with silt clay fraction. Their interaction with clay surfaces is probably the reason for their accumulation and protection.

Conclusions

Quantity and quality of HC were strongly affected by the type of soil, showing a clear relationship with the aggregate pore size distribution, while no relevant differences were observed according to the type of fertilizer. Transient compounds found in sand soil, suggest the presence of limited humification processes and a restricted SOC protection capacity. Instead in clay and peaty soils it was found high aggregate porosity amounts in the range 5–0.0035 μm , evidencing relevant presence of SOC spatial inaccessibility protection mechanisms. In particular in clay soil it was found compounds likely to be protected by interaction with mineral surfaces and intercalation in phyllosilicates. Peaty soil had recalcitrant compounds in macro and microaggregates, suggesting SOC selective preservation processes. Although complex physical, chemical and biological processes and their

interactions govern the SOC turnover, there was an evidence that soil porosity distribution could be a valuable indicator of the soil capacity to sequester organic carbon. Considering our data we can suggest that SOC reserve can be efficiently enhanced and protected in clay, particularly in silt clay fraction and in microaggregates. Otherwise, for peaty soil, the silt clay fraction resulted C saturated (Chapter 1), allowing to increase SOC only in microaggregates, mainly due to physical protection. Sand soil resulted not suitable for carbon sequestration purposes.

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