

Chapter 7

The Soil Humeome: Chemical Structure, Functions and Technological Perspectives



Alessandro Piccolo, Riccardo Spaccini, Davide Savy, Marios Drosos and Vincenza Cozzolino

Abstract Humus or humic substances (HS) are of pivotal importance in the global ecosystem dynamics, since fluctuation in their amount affects not only the growth of both plants and soil microorganisms, but also the main biogeochemical cycles. The development of technologies aimed at controlling HS in the agroecosystem processes is hindered by the limited knowledge of their chemical structure and dynamics. The recent acknowledgement of the supramolecular nature of soil HS allowed to devise a fractionation procedure, called Humeomics, that enables a detailed characterization of the structure of humic molecules in soil. Humeomics produces homogeneous fractions by progressively breaking esters and ether C–O bonds but not carbon–carbon bonds. The molecules in fractions are then identified by means of advanced spectroscopic and mass spectrometric techniques, thereby providing a body of structures that may well represent the soil Humeome. Humeomics enabled to unravel the effects of different soil management practices on soil carbon dynamics and to explain the recalcitrance of HS in soil. Moreover, the application of Humeomics allowed to corroborate the novel concept of humification, that is unambiguously described as the progressive accumulation of hydrophobic molecular components, which are no longer biotically accessible, due to their rapid thermodynamically driven partitioning from liquid to the solid soil phases. Conceiving HS as supramolecular associations of relatively small compounds also helped to unravel the reactivity of HS with respect to plant and microbial development, as well as towards xenobiotics. Finally, the supramolecular understanding of HS encouraged the proposal of an innovative technology for the control of organic matter stabilization in soil. This is based on the in situ photo-polymerization of humic molecules catalysed by metal porphyrin biomimetic

A. Piccolo (✉) · R. Spaccini · V. Cozzolino

Interdepartmental Research Centre on Nuclear Magnetic Resonance for the Environment, Agro-Food and New Materials (CERMANU), University of Napoli Federico II, Portici, Italy
e-mail: alessandro.piccolo@unina.it

Department of Agricultural Sciences, University of Napoli Federico II, Portici, Italy

D. Savy

Plant Biology Laboratory, University of Liège, Gembloux Agro-Bio Tech, Gembloux, Belgium

M. Drosos

Faculty of Biology and Environment, Institute of Resource, Ecosystem and Environment of Agriculture (IREEA), Nanjing Agricultural University, Nanjing, China

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catalysts. The resulting increase in the molecular mass of humic molecules was found not only to increase soil aggregate stability but also to sequester in soil significant yearly amounts of organic carbon. It is expected that the research findings presented here will prompt novel studies on the man-driven control of the soil Humeome in order to increase its content in soil, and contribute to positively affect both crop yields and soil microbial activity.

Keywords Humus supramolecular structure · Soil Humeome · Humeomics · Humification · Recalcitrance of soil carbon · Carbon sequestration

7.1 Introduction

Soil organic matter (SOM) can vary greatly with soil properties. Though SOM content is generally low in agricultural soils, it is nonetheless a key factor in determining the fertility of soil and the yield and quality of crops (Wood and Baudron 2018). SOM represents the largest carbon pool on Earth, approaching 1,300 Pg C in the upper 1 m and 3,000 Pg C when including deeper soil carbon (Köchy et al. 2015). SOM is composed of up to 80% by humus or humic substances (HS), which are organic materials ubiquitously found in soils, whose reactivity and dynamics are directly linked to soil quality and microbial activity, as well as to the soil C sequestration capacity and plant productivity (Canellas and Olivares 2014; Woo et al. 2014). HS have been traditionally and operationally defined by their solubility in water at different pH values: fulvic acids (FA), soluble under all pH values; humic acids (HA), insoluble at pH value < 2; and humin, insoluble at any pH value (Stevenson 1994). It is important to note that intensive agricultural practices have determined an alarming acceleration in HS mineralization, thus leading to both an increase in greenhouse gases emission from soils and a degradation of soil quality (Smith et al. 2014). Hence, preserving or even increasing the amount of soil HS in soil is essential for keeping high crop yields (Canellas and Olivares 2014) and sequestering carbon in soil (Piccolo 2012). This task can be adequately reached only once, and a thorough understanding of the molecular nature of HS is attained, in order to then wittingly and profitably intervene on their dynamics and relationships with both soil microbiota and plant roots.

7.2 Humic Substances, Chemical Composition and Physicochemical Properties

The scientific community of humic scientists has tried to unravel HS structure since the eighteenth century, when Achard (1786) for the first time isolated and fractionated peat HS. The difficulties met in precisely describing the structures and reactivity of HS, due to the HS chemical complexity and heterogeneity continued even when more

sensitive and powerful analytical techniques have become available. In fact, since HS arise from the decay of plant tissues and microbial metabolites, their molecular heterogeneity is difficult to unravel. Only at the end of last century, the real nature of the HS began to be clarified (Piccolo 2001, 2002). Before then, HS were described as macropolymers, with dispersive molecular masses from ten to hundred thousand daltons. However, all efforts to isolate and characterize such macropolymers failed together with the provision of unequivocal evidence for the occurrence of any polymerization process in natural soil systems (Hayes 2009 and references therein).

Only in the mid-1990s, the supramolecular nature of HS has been called upon to account for the conformational behaviour of HS, based on the results of, first, low-pressure size-exclusion chromatography (Piccolo et al. 1996) and, then, by high-performance size-exclusion chromatography (HPSEC). Aqueous HS solution that was injected in the HPSEC system before and after the addition of very small amount of acetic acid (AcOH) showed significant changes in the elution profile (Conte and Piccolo 1999; Cozzolino et al. 2001). In particular, lowering the solution pH value from 7.0 to 3.5 produced a hypochromic effect (a dramatic reduction in peak absorbance) for some elution peaks, which were concomitantly shifted towards larger elution volumes. This phenomenon could only be explained with a new understanding of the humic molecular structure: a supramolecular association of heterogeneous and relatively small molecules (not more than 400–1000 Da) self-assembled by weak dispersive forces (van der Waals, $\pi-\pi$, $\pi-\text{CH}$) into apparently large molecular sizes. In fact, the pH value change due to the AcOH addition to the loosely bound humic suprastructures causes the formation of new intra- and intermolecular hydrogen bonds and disruption of the weak dispersive forces which metastabilize the humic clusters at pH value of 7.0 (Cozzolino et al. 2001; Piccolo 2002). The result was the separation of the suprastructures into smaller but more stable associations showing lesser absorbance of incident electromagnetic radiation. Further evidence was obtained by repeating the experiment with dioic acids, namely oxalic, malonic, succinic and glutaric acids, which should have mimicked the effects of commonly root-exuded organic acids on HS conformations (Piccolo et al. 2003a). The authors reported modifications in the HPSEC profiles comparable to those observed when adding AcOH. The employed dioic acids had progressively longer carbon chains corresponding to different pK_a values and increasing degrees of protonation at pH 3.5. Hence, concomitant to the formation of new strong hydrogen bonds, a better contact with the hydrophobic moieties of the loosely bound humic superstructures was reached by the longer chain acids, thus causing a more extensive HS conformational rearrangement. The HPSEC thus proved to be an useful tool to assess whether a complex mixture displays a supramolecular or a polymeric nature (Piccolo et al. 2001), and it is still currently employed to confirm the supramolecular nature of humic-like materials from composts or lignin-like extracts from lignocellulosic biomasses (Maia et al. 2008; Savy and Piccolo 2014; Savy et al. 2016a, 2017). The novel understanding of the supramolecular nature of HS shed new light on their possible interactions with plant exudates and microbially derived soil molecules, and provided plant physiologist better chemical bases to unravel the mode of action of HS towards plant nutrition (Canellas et al. 2008).

The conformational changes observed by HPSEC were also confirmed by diffusion-ordered nuclear magnetic resonance spectroscopy (DOSY-NMR). This technique was applied to several FA and HA in order to measure their diffusion coefficients (D) (Šmejkalová and Piccolo 2008a, b). An inverse correlation between FA and HA concentration and the corresponding D values were noted and explained with the capacity of humic molecules to self-assemble with increasing concentration, thus concomitantly decreasing the diffusion of larger associations. On the other hand, when the pH value of the humic suspensions was lowered down to 3.6, the DOSY-NMR spectra showed a significant increase in diffusivity due to the disruption of large assemblies in more rapidly diffusing smaller aggregates, thus proving also by this technique the supramolecular structure of both FA and HA (Šmejkalová and Piccolo 2008a, b).

7.2.1 Literature Evidence of the Supramolecular Structure of HS

Once the traditional paradigm of the polymeric nature of HS has been challenged by the supramolecular view, a series of experiments have been carried out, in order to verify the validity of the hypothesis postulated by Piccolo and co-workers. Varga et al. (2000), for example, suggested that the dramatic modifications of pH value in the HPSEC columns, as obtained by adding organic acids, may provide misleading results. Hence, in response, Piccolo et al. (2001) compared the HPSEC profiles of HS with those of real polymers of well-known molecular mass (namely, polysaccharides and polystyrene sulphonates) and showed that the real polymers added with AcOH did not show any change in HPSEC behaviour as it did in the case of HS, thereby showing that only metastable humic conformations can be disrupted by organic acids additions and not those of covalently stabilized polymers. Simpson (2002) analysed the DOSY diffusivity of HA at two different concentrations and also found a negative correlation between humic concentration and diffusivity. He explained the observed results by invoking the ability of organic acids of causing a modification of the supramolecular humic conformation (Simpson 2002), thus supporting the evidence brought about by Piccolo (2001, 2002).

The supramolecular concept of HS served to explain the behaviour of HS in various experiments. For example, Peuravuori and Pihlaja (2004) and Peuravuori (2005) studied HS from freshwaters derived from spodosol watersheds by both HPSEC and NMR spectroscopy. They showed that the humic substrates were actually composed of nano-scaled supramolecular associations of structurally similar compounds of various ranges of molecular sizes.

Furthermore, two-dimensional NMR spectroscopy and transmission electron microscopy were applied to study the structure of commercial and river water HS in solution as a function of cation type and concentration, pH and solution salinity (Baalousha et al. 2005, 2006). Results were explained by considering HS as

associations of relatively small-sized compounds, interconnected in more complex assemblies by dispersive forces and through cation binding.

A plausible explanation for the interaction of metals with humic compounds was reached when the HS supramolecular structure was introduced. Buurman et al. (2002) analysed the thermal stability of soil HS saturated with H, Na, Ca, or Al and after a treatment with either methanol or formic and acetic acids. Interestingly, the thermal behaviour of protonated humic molecules (i.e. the control material) did not show any difference upon addition of polar organic molecules, while the thermal oxidation of Na-humates was shifted to larger temperatures than control. Moreover, a significantly lower effect of the polar compound addition was recorded for Ca-humates, whereas *non*-significant modifications of the thermal characteristics of Al-humates were noted. According to the authors, the humic components were strongly bound to each other by hydrogen bonds in protonated HS and by electrostatic bridges in Ca- and Al-humates. Such binding forces could not be overcome by adding polar organic molecules, thereby maintaining the stability of such substrates which could be only slightly altered. Conversely, in Na-humates, humic molecules were held together only by *non*-specific weaker hydrophobic interactions, which could be altered by adding polar organic molecules. It is noteworthy that such a different thermal behaviour of the metal-complexed humates can be hardly explained by considering HS as macromolecular polymers, while it is fully understandable by conceiving HS as supramolecular assemblies (Buurman et al. 2002).

Other workers exploited a wide array of analytical techniques for the characterization of the aggregates formed by supramolecular humics and hydrolysed Fe species under several pH values and mixing conditions (Siéliéchi et al. 2008). At low Fe concentration, the humic molecules self-organized into compact structures, while at increasing Fe amount the rearrangement of humic colloids was significantly reduced. The authors concluded that the interaction between HS and Fe ions led to a competition between the rearrangement of the humic network and the collision rate of metastable colloids. Again, these results were only explained by assuming HS as small self-associated molecules, rather than real polymers (Siéliéchi et al. 2008).

These conclusions were corroborated by the work of Nuzzo et al. (2013), who reported a decreasing molecular weight (MW) distribution of HA when they were progressively complexed with increasing Fe amount. The authors explained their findings by the disruption of the weak hydrophobic bonds stabilizing the humic conformational structures by the strong iron complexation that forced the humic superstructures into smaller-sized aggregates but of greater conformational stability.

Furthermore, Nebbioso and Piccolo (2009) studied the mobility and the diffusivity of Al- and Ca-humates by measuring the spin–lattice relaxation time in the rotating frame ($T_{1\rho}(H)$) by cross polarization magic angle spinning (CPMAS NMR) spectra, and the diffusion constants (D) by DOSY-NMR spectroscopy, respectively. Results suggested that the molecular rigidity of HA significantly increased with metal addition throughout the full carbon spectral region, and that it was more pronounced for triple-charged Al ions than for double-charged Ca ions. The $T_{1\rho}(H)$ values of the various spectral ranges indicated that the increase in molecular rigidity followed the order: aliphatic C > aromatic/double bonds C > carboxyl C. DOSY spectra also

showed that complexing HA with Al^{3+} or Ca^{2+} resulted in lowering D values for alkyl components, while concomitantly increasing those for aromatic and hydroxyl-alkyl moieties. The findings suggested that metal complexation prompted a molecular size increase in the former and a decrease in the latter, thus pointing out the preferential role of saturated and unsaturated long-chain alkanolic acids to be involved in complexation with Al and Ca, with a consequent increase in conformational rigidity and molecular size of humic hydrophobic domains. On the contrary, the more hydrophilic or mobile humic components seemed to be relatively less affected by the rearrangements of humic molecules induced by metal complexation (Nebbioso and Piccolo 2009).

7.2.2 *The Question of the HS Molecular Mass*

Since HS were no longer regarded as polymers, questions were raised about their real molecular size. In fact, before being aware of their supramolecular nature, scientists had proposed that HS molecular mass could be up to several hundred thousand Da (Stevenson 1994), whereas the real size of humic molecules may be much less. A useful technique to investigate this point appeared the modern liquid chromatography hyphenated with mass spectrometry. The electrospray ionization interphase to mass spectrometry (ESI-MS), being a soft ionization process, has the advantage of providing unfragmented ions, thus helping to obtain not only an absolute MW value but also an unambiguous information on the chemical structure of the analysed molecules when the MS/MS mode is applied to humic samples (Nebbioso et al. 2010).

By using a low-resolution ESI-MS instrumentation, Piccolo and Spiteller (2003) showed that the average molecular size of a soil humic acid was much lower than 1,000 Da. Furthermore, sample heating by changing the ESI cone voltage was found not to produce any molecular fragmentation, thereby implying that ESI-MS enabled the assessment of the real mass of humic molecules (Peuravuori et al. 2007; Piccolo et al. 2010). However, the size fractions separated by a preparative HPSEC from the same soil humic acid used by Piccolo and Spiteller (2003) showed similar molecular sizes as the unfractionated HA, thus suggesting that the heterogeneous supramolecular associations of humic molecules responded differently to the ESI ionization. The ESI-MS technique may then have some limitation in adequately ionizing all molecules present in complex hydrophobic and hydrophilic systems, and such constraints can explain the similarities in the average molecular mass noted for both fractionated and unfractionated HA. In fact, Nebbioso et al. (2010) found that a mixture of both polar and apolar molecules had a heterogeneous electrospray ionization, depending on their most probable positioning at the surface of the evaporating droplet during the electrospray ionization process. Since hydrophobic compounds are most likely positioned at the aqueous-gas interphase, the ionized apolar components of the droplet are preferentially transferred in the gas phase, as compared to the more polar molecules, which are instead preferably retained in the inner part of the droplet and

never reach the droplet surface before it is discarded. Hence, the ESI-MS detection of hydrophobic compounds is comparatively larger than for hydrophilic molecules.

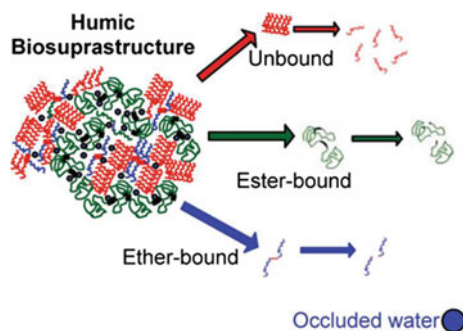
The role of hydrophobic molecules in stabilizing humic biosuprastructures was pointed out by several studies. Chilom et al. (2009) reported that the apolar-containing moieties play a key role in both initiating the aggregation process and facilitating the formation of micelle-like structures. Instead, Conte et al. (2006, 2007) separated by preparative HPSEC several HA fractions of decreasing molecular size which were then characterized by advanced physical–chemical techniques. By applying the variable contact-time pulse sequence of CPMAS NMR spectroscopy, the authors were able to calculate both the cross polarization (T_{CH}) and the proton spin–lattice relaxation ($T1\rho H$) times for the separated size fraction, and related such parameters to structural differences among the various fractions. They found that larger size fractions were characterized by a greater number of hydrophobic domains with slower molecular motion, whereas the smaller size fractions contained more polar moieties, which showed faster local molecular motion. These results were in line with the supramolecular understanding of humic matter and confirm that the apparently large sizes are stabilized by dispersive bonds among hydrophobic components.

7.2.3 *Humeomics and Humeomes*

Conceiving the HS as associations of relatively small molecules (<1,000 Da) held together in only apparently large molecular sizes by weak dispersive forces, such as hydrogen bonds, hydrophobic intermolecular interactions and metal-bridged intermolecular electrostatic linkages, led to develop novel procedures to separate humic superstructures into less complex and more homogeneous sub-fractions, in order to facilitate their structural identification (Fiorentino et al. 2006; Spaccini and Piccolo 2007; Nebbioso and Piccolo 2011, 2012; Drosos et al. 2018a). The new chemical fractionation of humic matter coupled to the advanced spectroscopic and chromatographic techniques for the characterization of humic molecules was called Humeomics, similarly to other -omics approaches, and had the aim to separate the single humic components from the bulk matrix without breaking any C–C bond. Hence, the overall humic molecules separated and identified by Humeomics have been grouped under the term Humeome (Monda et al. 2018). Humeomics has been described as “a stepwise separation of molecules from the complex bulk suprastructure by progressively cleaving esters and ether bonds and characterizing the separated molecules by advanced analytical instrumentation” (Fig. 7.1, Nebbioso and Piccolo 2011).

The Humeomics fractionation (Fig. 7.2) entails first of all a treatment of the bulk HS (also called RES0) with a mixture of organic solvents (dichloromethane and methanol), in order to isolate a fraction called ORG1 composed of free and unbound humic compounds, which are associated with each other only by weak dispersive forces. Then, the readily accessible ester linkages of the Humeome are transesterified by means of a BF_3/CH_3OH solution, and the resulting supernatant is separated

Fig. 7.1 Conceptual approach for the Humeomics sequential fractionation (Nebbioso and Piccolo 2011)



into dichloromethane- and water-soluble fractions (called ORG2 and AQU2, respectively), obtained by liquid–liquid separation of the transesterified product. A further cleavage of ester linkages with a KOH/CH₃OH mixture allows the extraction of those ester-bound compounds that were not physically accessible during the first transesterification. Again, the hydrolysed components are separated into organosoluble (ORG3) and hydrosoluble (AQU3) fractions. In the last step, the remaining humic matrix is treated with a 47% HI aqueous solution, in order to cleave ether bonds, yielding again both organosoluble (ORG4) and hydrosoluble (AQU4) components. The remaining solid residue (RES4) can then be finally treated with a traditional alkaline solution to solubilize the *non*-extractable humic compounds.

A complete Humeomics fractionation was first performed to reach the molecular composition of HA extracted from a volcanic soil (Nebbioso and Piccolo 2011). The bulk RES0 was studied by high-resolution Orbitrap electrospray (ESI) mass spectrometry after elution through a hyphenated HPSEC column. The preliminary separation of this Humeome by HPSEC simplified the Orbitrap mass spectra and allowed to ascertain that RES0 was mainly composed by alkanedioic and hydroxydioic acids. For example, alkanedioic acids, di- and tri-hydroxylated C18 acids, monounsaturated C14, C16 and C18 acids, hydroxy-unsaturated C6–C20 acids, C4–C24 dioic, C16–C24 hydroxydioic, cyclic acids were found in the RES0 fraction with large elution time. In the same elution interval, a cyclic acid with empirical formula C₇H₆O₈ was also identified, and, due to its large unsaturation, it was suggested that it had an aromatic character, such as a hydroxylated and carboxylated furan ring. Conversely, the still great molecular aggregation in the HPSEC fraction eluted at smaller elution time prevented the correct assignment to any definite empirical formula or class structure (Nebbioso and Piccolo 2011).

The ORG1 fraction, comprising the unbound organosoluble humic components, was analysed by both gas chromatography-mass spectrometry (GC-MS) and NMR spectroscopy. The two techniques showed that this fraction contained a predominance of alkyl and saturated components, particularly mono- and dicarboxylic acids and some iso- and anteiso-branched alkanedioic acids and hydroxyacids, such as ω-C16-18, β-C14 (Nebbioso and Piccolo 2011).

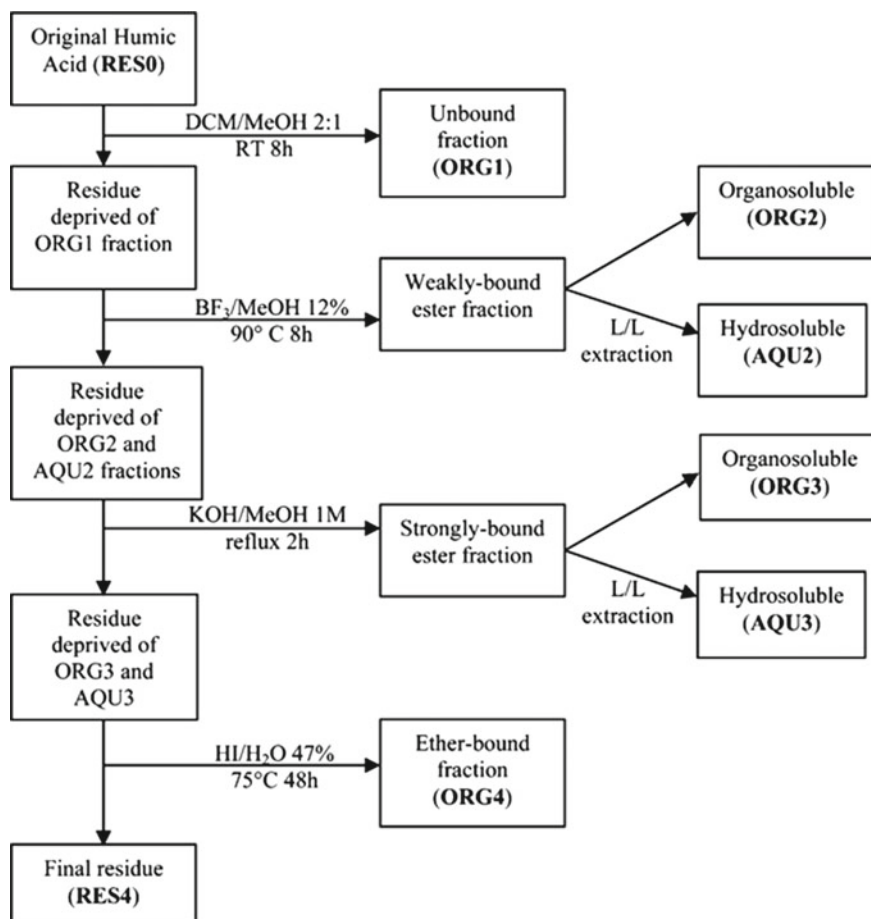


Fig. 7.2 Scheme of Humeomics fractionation (Nebbioso and Piccolo 2011). M = mol L⁻¹

The transesterification with $\text{BF}_3/\text{CH}_3\text{OH}$ solution and the subsequent separation of the supernatant in its dichloromethane (DCM)- and water-soluble components produced the ORG2 and AQU2 fractions. The NMR spectrum of ORG2 showed that its chemical structure was similar to that of ORG1, while its GC-MS characterization revealed significant amounts of unsaturated C9-29 linear and branched alkanolic acids, α/β C9-26 mono- and di-hydroxyacids, C18 unsaturated acid, C8-30 α,ω -diacids, sterols, C12-28 *n*-alkanols, and substituted benzoic and cinnamic acids. It is important to note that such an abundance of both hydroxyacids and unsaturated compounds in ORG2 could have been unveiled only after the removal of the unbound alkanolic and saturated acids that were identified in ORG1 (Nebbioso and Piccolo 2011). The AQU2 contained less unsaturated compounds and larger oxygen substitution than in RES0 and the previous ORG fractions. Also, a large

content of *N*-containing compounds was reported and ascribed to the larger affinity of nitrogen-rich components for the aqueous phase. However, such fraction still contained a large amount of odd- and even-numbered C10-18 saturated acids, C15-18 unsaturated acids, C15-18 hydroxy-unsaturated acids and C5-9 diacids. Moreover, the ESI technique allowed identifying C7-18 negatively charged compounds containing one to five N atoms, as well as cyclic acids and $C_xH_{2y}O_y$ carbohydrate structures. Several other unidentified *N*-containing compounds were mostly represented by the $C_8H_7O_3N_3$ empirical formula (Nebbioso and Piccolo 2011).

The resulting residue was then subjected to another transesterification reaction, carried out with a KOH/CH₃OH mixture, in order to cleave the remaining ester bonds. A subsequent liquid-liquid extraction allowed a further separation of molecules according to their solubility in DCM or water. The NMR spectra of ORG3 showed several peaks attributed to either aliphatic or CH-X compounds, such as hydroxylated acids, and these findings were confirmed by GC-MS. The latter technique revealed the occurrence of C9-32 saturated and unsaturated *n*-alkanoic acids, C6-8 α,ω -diacids, C16,22,24 ω -monohydroxyacids, C22-26 β -monohydroxyacids, C18 di- and tri-hydroxyacids, C12-28 *n*-alkanols, phenolic acids and steroids. Also, two-dimensional NMR techniques (COSY, TOCSY and HSQC) were applied and suggested that ORG3 contained several aromatic and CH-X molecules. The small amount of the AQU3 fraction prevented any characterization of its composition (Nebbioso and Piccolo 2011).

Finally, the ether linkages in the humic residue were cleaved by means of a 47% HI aqueous solution, thus yielding ORG4 and a remaining solid residue, named RES4. While the former was not obtained in sufficient amount, the latter was enough to be studied by liquid- and solid-state NMR spectroscopy, as well as by high-resolution ESI-MS (Nebbioso and Piccolo 2011). Results showed that RES4 contained low amount of alkyl structures and was completely depleted of alkanedioic acids, as also revealed by measuring the proton relaxation time ($T1\rho$ H), that is an indicator of changes in molecular rigidity. Specifically, a tighter molecular packing was observed for RES4 fraction than for RES0. Furthermore, the solid-state dipolar-dephasing NMR technique indicated that RES4 contained a large amount of quaternary carbons, thus suggesting that humic matter remaining after Humeomics was largely formed by totally substituted or condensed aromatic moieties. Finally, Nebbioso and Piccolo (2011) solubilized RES4 in an alkaline solution and studied it by HPSEC-ESI-MS. Results revealed the presence of odd- and even-numbered C10-18 saturated acids, C14-18 unsaturated acids, C6,8 hydroxy-unsaturated acids and cyclic acidic structures. Overall, the proposed chemical fractionation allowed to identify up to 60% of the Humeome, while the lack of determination of the remaining 40% was attributed to loss of occluded hydration water, small volatile organic compounds, and/or decarboxylation reactions (Nebbioso and Piccolo 2011).

In order to further simplify the complex Humeome, Nebbioso and Piccolo (2012) size-fractionated by preparative HPSEC the same HA formerly studied by Humeomics, and the three separated fractions were again subjected to the Humeomics procedure. They identified a far larger number of compounds in the Humeome of the three size fractions than in that of the unfractionated HA. These results were

explained by the ability of the HPSEC preliminary separation to weaken the conformation of the humic superstructures with the consequent separation into less complex humic fractions, which can be then studied in further details by advanced analytical tools. The hydrophobic components were mainly in the largest size fraction, while the hydrophilic compounds were eluted in the smallest size fraction. Such results were in line with previous studies (Conte et al. 2006, 2007) and corroborated the supramolecular view of humic nature, according to which the intermolecular hydrophobic interactions are of pivotal importance in stabilizing the humic associations of apparently large sizes. The N-containing molecules were mostly present in the middle size fraction, possibly owing to their amphiphilic nature. Altogether, these results indicated that the conformation of supramolecular clusters was driven by the structural characteristics of the prevalent humic molecules. Interestingly, the lack of residual RES4 fractions in any of the size fractions corroborated the idea that the preliminary HPSEC separation enabled a reduction in the strength of the humic intermolecular association, thereby allowing Humeomics to fully dissolve the molecules composing the Humeome (Nebbioso and Piccolo 2012).

A size fractionation by preparative HPSEC was also adopted to further simplify the Humeome of the RES4 fraction, after its solubilization in an alkaline solution (Nebbioso et al. 2014a). Ten different size fractions were separated and then injected into an analytical HPSEC system hyphenated with a high-resolution Orbitrap ESI-MS. The resulting total ion chromatograms of the fractions showed two eluting peaks, whose empirical formulae could be mostly associated with linear alkanolic, unsaturated, hydroxylated, hydroxy-unsaturated acids and cyclic acids. Quantitative measurements highlighted the importance of long, saturated and unsubstituted linear acids in the stabilization of large supramolecular assemblies, their interaction leading to the formation of a more favoured packing among such compounds, compared with that arising by the interaction of irregularly shaped cyclic, unsaturated or hydroxylated compounds (Nebbioso et al. 2014a). Several unknown compounds had been also eluted by HPSEC, and they were studied by running tandem MS. The double fragmentation allowed to infer the nature of compounds whose m/z was 129, 141, 155 and 217, and whose empirical formulae were $C_6H_{10}O_3$, $C_6H_6O_4$, $C_6H_4O_5$ and $C_7H_6O_8$, respectively. The analysis of daughter ions helped to assign the ESI-derived peaks to hydroxy-unsaturated hexanoic acid, two furane rings and a norbornane-like ring. Importantly, the latter three compounds had never been reported before for terrestrial HA and recall the carboxyl-rich molecules already proposed earlier for dissolved organic matter (DOM) (Hertkorn et al. 2006). The size fractions obtained by subjecting RES4 to preparative HPSEC were further examined by several two-dimensional NMR techniques, which indicated that the chromatographic separation caused profound conformational modifications on the RES4 structure and provided simpler humic material. Consequently, less NMR signal overlapping in size fractions was noted, and more homo- and hetero-correlations were obtained for the size fractions than for the bulk RES4, showing mainly hydroxy-alkyl and alkyl protons (Nebbioso et al. 2014b). DOSY-NMR spectra confirmed the larger homogeneity reached by separating RES4 by HPSEC elution and allowed to recognize that nominally larger, lipid-rich size fractions had significantly lower constant

diffusivity, likely due to the formation of stable supramolecular associations, as promoted by hydrophobic interactions among alkyl chains. Instead, the diffusivity gradually increased in smaller-sized fractions, which contained a larger amount of aromatic and hydroxyaliphatic peaks and are not able to promote the formation of large suprastructures as much as alkyl-rich compounds (Nebbioso et al. 2014b).

Humin (HU) from a volcanic soil was characterized by Humeomics before and after a HF treatment, which was used to destroy the clay mineral components (Nebbioso et al. 2015). The obtained fractions were studied by GC-MS, thermochemolysis GC-MS and ^{13}C CPMAS NMR. Both weight and chromatographic yields were greater for the mineral-deprived HU than for bulk HU, hence, allowing better molecular identification in the Humeome of the former. Saturated and unsaturated alkanolic, ω -alkanedioic, hydroxyalkanoic acids, alkanols and hydrocarbons were identified in both HU samples. The large content of odd-numbered *n*-alkanoic acids found in ORG1 suggested an accumulation of free microbial metabolites, whereas plant-derived (even-numbered) alkanolic acids were predominantly recognized in both ORG2 and ORG3 fractions, implying an interaction of such compounds with the HU stronger than for microbial metabolites. Instead, unsaturated, *n*-alkanedioic and hydroxyalkanoic acids were noted only after hydrolysis of complex esters, i.e. in the ORG 3 fraction (Nebbioso et al. 2015). In line with previously reported findings for HA, the amount of aromatic compounds in HU residues progressively increased with Humeomics steps, while that of alkyl and hydroxy-alkyl molecules was reduced. The AQU2 extracts, in fact, were mainly composed of alkyl aromatic and carbohydrate-like compounds, while aromatic moieties were predominant in the RES fractions. Moreover, a thorough similarity in the identified compounds in both HA and HU isolated from the same soil further indicated that the differences among the traditional humic fractions (FA, HA and HU) resided more in the supramolecular arrangement of the contained molecules than in their molecular composition (Nebbioso et al. 2015).

Altogether, the above-described results confirmed that Humeomics enables to reach a deep and exhaustive molecular characterization of HS, thus allowing the isolation of molecules bound to the humic suprastructures by either weak dispersive forces or ester and ether linkages, without breaking carbon-carbon bonds. The progressive extraction of single humic molecules and their structural identification by cutting-edge physical-chemical analytical tools effectively advance the knowledge of the molecular composition of Humeome. In addition, Humeomics procedure may help to develop novel realistic computational models for Humeome conformation, in order to further relate its molecular structure to its biological activity towards both plants and microbes, thereby unravelling its environmental reactivity (Schaumann and Thiele-Bruhn 2011; Orsi 2014).

7.3 The Role of the Humeome in Soil Functions

The inherent molecular complexity and widespread diffusion of the soil Humeome determine the environmental importance of HS as keystone of SOM functions. In agricultural and forestry ecosystems, humic components play a crossroad influence in all the physical, chemical and biological processes associated with soil microbial–plant interactions and overall soil fertility.

7.3.1 Effect on Plant Growth and Soil Biological Fertility

The soil Humeome is a determinant factor for the maintenance of soil processes associated with plant growth and crop productivity (Olaetxea et al. 2018). In fact, besides their role in soil nutrient dynamics, humic molecules are involved in the biostimulation of plant development, acting as bioeffectors in a large range of biochemical activities, physiological processes and plant–microorganisms interactions (Canellas and Olivares 2017; Cozzolino et al. 2013). Therefore, an increasing effort is devoted to understand the effects of HS on plant metabolism and physiology, in order to support innovative technologies enabling the sustainable management of agro-ecosystems (Canellas et al. 2011, 2015).

The most important a-specific influence of the Humeome on plant growth is referred to the “*nutritional-environmental pathways*” (Olaetxea et al. 2018). This is related to the improvement in nutrient availability due to the effect of organic matter on the physical and chemical processes of soil rhizosphere (porosity, aggregation-texture, respiration, water availability and diffusivity, pool of bioavailable nutrients).

HS are recognized as important intermediates in the biogeochemical cycle of nitrogen, phosphorus and sulphur, as well as reservoir of these essential nutrients (Kelley and Stevenson 1996; Magid et al. 1996). A well-known effect is the increase in P availability induced by the adsorption of dissolved humic components on mineral surfaces of acidic soils and the concomitant displacement of P from iron and aluminium hydroxide. Furthermore, HS may interact with phosphate anions through the formation of metal bridges and enhance P solubilization and uptake (Urrutia et al. 2014).

HS form stable and soluble complexes with micronutrients, such as Fe, Cu, Mn and Zn, thus increasing the potential bioavailability of these elements in alkaline and calcareous soils, where these metal ions are otherwise insoluble and largely unavailable for plants (Chen 1996). In this respect, a recent work used two humic acids from a volcanic soil (HAs) and leonardite (HA1) to synthesize insoluble complexes with iron and simulated the effect of plant exudates on iron solubilization in the rhizosphere (Nuzzo et al. 2018). This was achieved by displacing iron from the iron–humate complexes with a solution of either a microbial siderophore (deferroxamine mesylate DFOM) or a mixture of organic acids such as citric, oxalic, tartaric and ketoglutaric acids. The variation in iron extractability between the two

iron–humates was attributed to both the humic molecular composition and the steric hindrance of the extractants. Iron was more easily released from complexes formed with the more flexible conformational structure of HAs rich in aliphatic carbon than the rigid conformation of iron complexes with HA dominated by aromatic carbon. This work showed that iron–humate complexes can be used to enhance iron nutrition of plants, whose exudates rich in organic acids, together with microbial siderophores, can displace iron from complexes and enhance its solubility in the rhizosphere.

Concomitantly, the same process may solubilize humic matter from metal–humates, thereby enhancing the concentration of bioactive humic molecules in the soil solution. In fact, humic matter from different sources may improve plant nutrition by activating the biochemical functions involved in nutrient root uptake, transport and metabolic cycles in plants (Piccolo et al. 1992; Vaccaro et al. 2009; Canellas et al. 2011). These HS-mediated effects were observed at both transcriptional and post-transcriptional levels (nutrient root transporters, proton pumps and key enzymatic activities) (Trevisan et al. 2010; Canellas et al. 2015). Therefore, the metal complexation by humic molecules can be complementary to the biochemical pathways in exerting the HS effect on plant growth (Olaexeta et al. 2018). In fact, humic extracts from either leonardite or composts were found to influence the expression of genes encoding the Fe(III) chelate reductase (CsFRO1) and the Fe(II) root transporter (CsIRT1) (Aguirre et al. 2009), and thus up-regulate the molecular mechanisms involved in Fe root uptake (Canellas et al. 2015).

The soil Humeome is involved in the stimulation of N assimilation by plant tissues and in the subsequent modulation of amino acid metabolism at both molecular and physiological levels (Vaccaro et al. 2015). Furthermore, a soil humic acid and its size fractions were found to induce a partial relief from response to phosphate (P_i) starvation in the cell culture of tobacco plants, thus increasing total cell phosphate amount, ATP and glucose-6-phosphate levels, as well as the activity of acid phosphatases (Zancani et al. 2009). Soil addition with a humic acid from vermicompost modified the expression of the high-affinity P root transporter gene in tomato plants (*LePT2*) (Jindo et al. 2016). In fact, in respect of control supplied with a low inorganic P_i concentration, plants added with HA showed a more efficient assimilation of available P sources and an increase in global phosphate uptake. These results indicated that the soil Humeome is strategically involved in plant adaptation to P availability through the activation of the metabolic pathway associated with the utilization of available P at low concentrations. Moreover, these findings confirm the complementarity of indirect and direct effects of HS on plant development and the relationships between biological processes and molecular properties of the soil Humeome (Nardi et al. 2007; Cozzolino et al. 2016a, b).

A direct beneficial action of HS on plant growth is related to their interaction with cell membranes of root surfaces (Nardi et al. 2017; Olaetxea et al. 2018). In fact, HS affect both the whole root growth, expressed by dry matter production, and the emergence and elongation of lateral roots and root hairs (Trevisan et al. 2010; Canellas et al. 2012; Canellas and Olivares 2017). It is believed that the biostimulation properties of HS are related to the increase in both the expression and activity of the plasma membrane (PM) H^+ -ATPase enzyme, with a consequent hormone-like

induction similar to that of auxinic phyto regulators (Dobbss et al. 2010; Canellas et al. 2011; Scaglia et al. 2016; Monda et al. 2018). Recent studies have further evaluated the mechanisms involved in the HS-dependent root development (Ramos et al. 2015). Plant treatment with a humic acid from vermicompost induced the emergence of lateral root in rice seedlings and specific H^+ and Ca^{2+} fluxes in the root elongation zone as activated by the plasma membrane H^+ -ATPase and the Ca^{2+} -dependent protein kinase (CDPK). The authors pointed out that the HA act as molecular elicitors of H^+ and Ca^{2+} fluxes, which seem to be the upstream of a complex CDPK cell-signalling cascade (Ramos et al. 2015). Currently, the HS enhancement of PM- H^+ ATPase functions observed in many crops is associated with the stimulation of integrated hormone crosstalk systems, which include, besides the auxin synthesis, the nitrous oxide (NO) and abscisic acid (ABA) metabolic pathways (Mora et al. 2010; Olaetxea et al. 2018). HS may also regulate the amount of reactive oxygen species (ROS) at the cellular level, as well as the expression of superoxide dismutase (SOD) genes in the cytosol (cCu/Zn-SOD1 and cCu/Zn-SOD2). This regulation of ROS (H_2O_2 and O_2^-) control by humic molecules occurs in the root regions involved in cell elongation and differentiation, thereby indicating a possible synergic effect of ROS in the HS-mediated root development (García et al. 2016).

Although the main HS action as bioeffectors is mediated by the interaction with root systems, the biostimulant activities of Humeome may affect the physiological processes linked with the development of shoot and leaves tissues in various crop species (Zancani et al. 2011; Canellas and Olivares 2014; Vaccaro et al. 2015). The application of soil humic acid and derived size fractions in nutrient solutions of maize seedlings showed a positive effect on plant metabolism, with a clear influence on the enzymatic steps of both glycolytic and Krebs biochemical cycles in plant leaves (Nardi et al. 2007). In distinct bioassay tests on maize, the use of different Humeome components isolated from various green composts revealed a steady positive correlation with the chlorophyll content of maize leaves combined with a significant increase in fresh and dry weight of the whole plant biomass (Monda et al. 2017; 2018). The increase in nitrate uptake observed in cucumber plantlets treated with a solution of humic acids was found to be coupled with an active translocation of cytokinins from root to shoot tissues and with a subsequent large mobilization of K, Ca and Mg in leaves (Mora et al. 2010; Rose et al. 2014). The HS-induced growth of shoots in cucumber seedlings was related to an increase in root hydraulic conductivity and K fluxes, determined by the ABA-dependent pathways that regulate plasma membrane aquaporin activity in roots (Olaetxea et al. 2015).

Most research works on HS bioactive properties are conducted in confined and simplified environments under controlled conditions and preliminary selected variables, thereby allowing the identification of specific biochemical and metabolic processes. On the other hand, the HS effects in real agroecosystems are probably the result of many interconnected biological and chemical processes and dependent on the simultaneous interaction of the dynamic supramolecular structure of the Humeome with soil microbiota and plant species (Nardi et al. 2017; Piccolo et al. 2018a). Part of the beneficial effects of soil humus on plants stems from the interaction between microbes and organic matter. Microbes behave as mediators of carbon

and nutrient turnover of soil humus, while the Humeome affects the survival and efficiency of microorganisms (Insam 1996; Lovely et al. 1996). In fact, the organic humified components influence both composition and activity of microbial communities of agroecosystems (Puglisi et al. 2013; Cozzolino et al. 2016a, b) with specific alternative effects, favouring either the selective preservation of beneficial microorganisms (Martinez-Balmori et al. 2013; Canellas and Olivares 2017) or an effective suppressive capacity of pathogens for different crops (Pane et al. 2011, 2013).

Therefore, additional complementary approaches on the study of bioactive properties of humic substances should be focused on the relationships between Humeome characteristics, and biological functions and cropping systems (Puglisi and Trevisan 2012; Ventorino et al. 2012; Scotti et al. 2016). In a bi-annual field experiment, the modification of SOM composition and biological activities attained by soil treatments with green compost produced a combined biostimulation effect on tomato plants with a significant improvement in nutrient uptake, fruit yield and quality (Pane et al. 2015). Furthermore, the inoculation of humic extracts and selected beneficial microbial species may promote a recovery of biochemical efficiency in crops grown under biotic and abiotic stress (Aguiar et al. 2016; da Piedade Melo et al. 2017).

The combined application to soil of compost and plant growth-promoting bacteria was tested as valuable and sustainable alternative management to the conventional phosphate mineral fertilization methods. The effects of the inoculation with different microbial isolates on the growth and metabolic processes of maize plants were evaluated in soil treated either with mineral fertilizers (triple superphosphate and rock phosphate) or with different humified manure-based compost as P-fertilizers (Li et al. 2017; Vinci et al. 2018a, b). Plants inoculated with organic materials and microorganisms showed larger P and N contents and a more differentiated metabolome as compared to the treatments with inorganic fertilizers. The metabolomic analyses revealed a significant increase in glucose, fructose, alanine and GABA metabolites in maize leaves, thus suggesting an improved photosynthetic activity due to enhanced P and N uptake. The inoculation with humified organic P-fertilizers sustained plant growth and the activity of microbial species and appeared as an efficient alternative to mineral fertilizers to enhance nutrients uptake and ensured plant growth under deficient soil P availability (Li et al. 2017; Vinci et al. 2018a, b).

Although the specific mechanisms underlying the action of humic substances as bioeffectors have not yet been completely elucidated, it is ascertained that the molecular characteristics and the structural properties of the Humeome have an influence on the plant activity and soil biological functionalities (Cozzolino et al. 2016a; Piccolo et al. 2018a). The supramolecular conformation based on the dynamic interaction of hydrophobic and hydrophilic domains was found to determine the bioactive properties of humic molecules in either exogenous organic inputs or SOM pools (Piccolo et al. 1992, 2018a; Piccolo 2002; Canellas et al. 2012). The hydrophobic composition of humic superstructures protects from microbial degradation the bioactive low molecular weight molecules of SOM, such as lignin fragments, phenol derivatives, carbohydrates, peptides and aromatic acids (Piccolo 2012; Muscolo et al. 2013; Piccolo et al. 2018a). In fact, the large concentration of root exudates and microbial products in the rhizosphere favours the physical–chemical interactions with the

metastable supramolecular structures (Canellas et al. 2008; Nardi et al. 2017). The flexible Humeome conformation may hence undergo a favourable thermodynamic rearrangement with a consequent release of the retained molecules which may exert their bioactive properties on root membranes and microbial cells (Piccolo 2002, 2012, 2016). The hydrophobic/hydrophilic ratio and the structural modifications induced by the physical–chemical interaction in the rhizosphere have been identified as the key structural processes of the bioactive properties of the Humeome from soil, sediments and recycled biomasses (Puglisi et al. 2008; Dobbss et al. 2010; Aguiar et al. 2013; Martinez-Balmori et al. 2014; Piccolo et al. 2018a).

The effects of a humic acid and its size fractions on plants carbon deposition and the structure of microbial communities were analysed in the soil rhizosphere of maize plants with rhizo-box systems, which allowed the simultaneous but distinct characterization of both bulk rhizosphere and plant roots exudates (Puglisi et al. 2009). Following the interaction with root exudation, all humic treatments were able to modify the structure of the rhizospheric microbial communities. However, while the small-sized and more bioavailable humified fractions provided a prompt response to the microbial activity, both the bulk HA and the most hydrophobic fractions required additional plant rhizodeposition before their bio-transformation released additional metabolic carbon.

The positive effect on maize development of different water-soluble Humeomes isolated from green composts was attributed to their significant hydrophobicity that favoured adhesion to the root surfaces. Then, exudation of organic acids from plant roots fosters the subsequent modification of the humic conformation and the concomitant release of bioactive molecules (Monda et al. 2017, 2018). However, in the same experiment, the large content of soluble phenolic compounds in the Humeome from tomato- and artichoke/fennel-based composts revealed either a small stimulation or even toxic effects on roots growth. The biostimulation was recovered at larger Humeome supply rates, and this was explained with a conformational tightening due to the hydrophobic components, whose closer intermolecular aggregation prevented the release of soluble toxic components (Monda et al. 2018).

In order to provide evidence for the Humeome structure–activity relationship, the effect of root exudation on the bulk Humeome superstructures was simulated with different mild chromatographic and physical–chemical fractionation procedures (Nardi et al. 2007; Puglisi et al. 2009; Vaccaro et al. 2009; Canellas et al. 2011, 2012). For all tested humic materials, results revealed that the more flexible the bulk Humeomes, the larger was the release of low molecular weight components and the larger the stimulation of biochemical and metabolic processes. The bioactivity of Humeome thus appears to be closely related to its molecular composition and consequent stability of its conformational structure in solution, whose dynamic interaction with rhizosphere determines the potential release of bioactive molecules that directly or indirectly influences plant growth.

The understanding of the bioactive properties of humic substances may support the adoption of alternative technologies to develop effective sustainable SOM management of agroecosystems. The current increasing demand of renewable energy based on the exploitation of biomasses, such as non-food crops, agro-industrial by-products

and organic wastes, leads to the increased availability of secondary products. Those left over from biorefinery processes include large amounts of lignocellulose residues which are currently burnt to produce thermal energy, with a net loss of photosynthetic organic carbon that may be rather employed in the sustainability of agricultural production.

To this purpose, a specific alkaline oxidative extraction was applied to lignocellulosic biorefinery wastes and provided significant yields of water-soluble derivatives enriched in lignin components (Savy and Piccolo 2014). The detailed characterization of these organic extracts revealed that they may act as humic-like substances (HULIS) which retain the molecular features and the bioactive properties of the natural Humeome (Savy et al. 2016b, 2017). Therefore, bioenergy crops and biorefinery wastes should be regarded as valuable source of natural plant biostimulants, either by direct extraction procedures (Savy et al. 2016a, 2017) or after their use as humic precursors in composting processes (Spaccini et al. 2019).

The water-soluble HULIS derivatives isolated from either lignocellulose biorefinery residues or lignosulphonate as wastes of paper mill industries were evaluated for their molecular characteristics and bioactivity on maize seedlings (Savy et al. 2017, 2018). All these materials showed a positive influence on plant growth, with different responses depending on specific structural composition and concentration rates. As found for the soil Humeome, the biostimulation effects were attributed to the humic-like conformational behaviour that determined the release of bioactive lignin-derived phenolic compounds.

A direct extraction of HULIS bioactive components from lignin-rich biomasses was applied to four non-food bioenergy crops: cardoon (CAR), eucalyptus (EUC), and two black poplars (RIP) and (LIM). After a detailed characterization of the molecular components of extracts, the potential bioactivity towards seed germination and early growth of maize plantlets was evaluated (Savy et al. 2015). These lignin-derived HULIS stimulated the maize seedling development, though to a different extent depending on the hydrophobic/hydrophilic properties of humic-like molecules. The most significant bioactivity, found in CAR-derived extracts followed by RIP and LIM treatments, was related to a large content of aliphatic OH groups, CO carbons and smallest hydrophobicity, as assessed by ^{31}P -NMR and ^{13}C CPMAS NMR spectroscopies. The poorest biostimulation of EUC HULIS was attributed to its smallest content of polar OH groups and largest hydrophobicity. Both these features may be conducive of a EUC conformational structure tight enough to prevent its alteration by organic acids exuded from vegetal tissues. Conversely, the more labile conformational arrangements of the other more hydrophilic lignin extracts promoted their bioactivity by releasing biologically active molecules upon the action with exuded organic acids.

In a corresponding study, water-soluble HULIS lignins were isolated from the most representative non-food bioenergy crops such as giant reed (AD) and miscanthus (MG) (Savy et al. 2016a, b). The extracts were characterized for their conformational structure and molecular composition employing size-exclusion chromatography and NMR spectroscopy. Their effects at different concentration rates on germination of maize seeds and growth of maize plantlets were assessed concomitantly

in growth-chamber experiments. HULIS showed both humic-like supramolecular structures and different conformational stability and molecular composition. Their largest bioactivity was revealed at 10 and 50 ppm of OC concentrations, with a significant increase in roots and coleoptiles length of maize seedlings, as well as of total shoot and root dry weights and root length of maize plantlets. Again, the differences in bioactivity of HULIS from AD and MG were attributed to their conformational stabilities and content of amphiphilic molecules, which may control both the adhesion to plant roots and the release of bioactive molecules upon interactions with plant-exuded organic acids.

7.3.2 *Effects on Soil Stability*

The physical properties of soil closely depend on the interaction with the soil Humeome. In fact, humus is involved in soil aggregation processes at different hierarchical levels, from the formation of organo-mineral complexes up to the stabilization of large size aggregates (Oades and Waters 1991). The humic molecules are the basic cementing agent of stable microaggregates which represent the resilient core of soil aggregate dynamics (Piccolo and Mbagwu 1999; Six et al. 2004). Humic substances promote an effective association of fine soil particles into larger aggregates, based on their hydrophobic components which exert a more permanent physical stabilization than the temporary and transient aggregation provided by fresh and biolabile plant inputs and microbial exudates (Piccolo and Mbagwu 1999; Spaccini and Piccolo 2012; Sarker et al. 2018; Piccolo et al. 2018a). The water-soluble components of the Humeome can perform a variable range of reversible chemical interactions with polar or charged mineral surface, like electrostatic interactions, ligand exchanges, cation bridges, hydrogen bonding, etc. (Nebbioso and Piccolo 2013; Paul 2016). The divalent Ca^{2+} and Mg^{2+} cations are believed to play a role for the initial mutual approaches of organic and mineral surfaces of clay–Humeome complexes in neutral and alkaline soils (Sutton and Sposito 2006), whereas the adsorption of HS occurs on the surface of iron and aluminium oxides in acidic soils (Oades 1988; Song et al. 2013).

The interaction of hydrophobic molecules with inorganic components enables a long-term stabilization to the soil structure (Piccolo and Mbagwu 1999). The lower affinity of dissolved hydrophobic compounds for the soil aqueous solution enhances the chemical potential towards the molecular adsorption on the available apolar and low-charged mineral surfaces (Schwarzenbach et al. 2003a; Feng et al. 2005; Nebbioso et al. 2015). This thermodynamic process is further increased by the small volume of the soil porous system, the effect of ionic strength of dissolved salts (e.g. “salting out” effect) and the presence of soluble organic components (Schwarzenbach et al. 2003a). The progressive accumulation of humic molecules constitutes an hydrophobic environment in soil that becomes tightly impermeable to the soil aqueous solution irrespective to pH values (Piccolo and Mbagwu 1999; Piccolo 2012; Masoom et al. 2016). This process leads to the progressive decrease in wettability of inorganic

surfaces, thereby increasing soil aggregate stability. This organic coating may thus strengthen the association between mineral particles and contribute to the stability of microaggregates by favouring interparticle aggregation by hydrophobic interactions (Piccolo et al. 2018a). Conversely, the highly hydrophilic biomolecules (carbohydrates and peptides) are more likely to partition into the aqueous soil solution, where they are rapidly degraded by microorganisms, thereby providing at best only a temporary effect on soil structural stability (Piccolo and Mbagwu 1999; Six et al. 2004).

The characterization of organic compounds associated with stable microaggregates in different soil types and land uses revealed the steady selective accumulation of alkyl hydrophobic molecules (Baldock and Skjemstad 2000; Piccolo et al. 2005a; Mao et al. 2007). The relation between soil aggregate stability and hydrophobic humic components was highlighted in heavily weathered African soils under original forest and agricultural management (Spaccini et al. 2002a, 2004). The aggregate size fractions derived from forest soils were characterized by a large structural stability and revealed an incorporation of highly hydrophobic humic materials (Piccolo et al. 2005a; Spaccini et al. 2006). The shift of land use with deforestation and cultivation produced a decrease in the hydrophobic portion of the soil Humeome with a concomitant progressive loss of large-sized water-stable aggregates and of overall physical stability.

7.3.3 *Effect on Carbon Sequestration*

The soil Humeome represents the most abundant SOC pool of forest and agricultural soils, acting as the bulk compartment for the incorporation, accumulation and stabilization of OM inputs (Piccolo 2012; Piccolo et al. 2018a). Despite the wide combination of ecological (soil type and texture, biological components, climate, vegetation) and anthropic (land use, management, cropping systems) that affects SOM dynamics, the soil Humeome from different ecosystems reveals comparable molecular characteristics and reactivity (Piccolo 1996; Drosos et al. 2017, 2018). This finding suggests the occurrence of general mechanisms which control the decomposition/accumulation reactions involved in the Humeome formation (Piccolo 2002; Piccolo et al. 2018a). The selective incorporation of less decomposable or persistent hydrophobic compounds, derived from the biotic and abiotic transformation of biomolecules released by dead cells, may be conceived as the driving stage underlying the humification process (Winkler et al. 2005; Lorenz et al. 2007; Nebbioso et al. 2015; Piccolo et al. 2018a). As noted above, the poorly soluble apolar substances undergo a favourable partition from the aqueous soil solution to the solid phase (Piccolo 2002; Schwarzenbach 2003a; Piccolo et al. 2018a). Organic molecules are hence adsorbed and retained on the surface of fine secondary minerals by non-covalent dispersive forces (Kleber et al. 2007; Paul 2016). However, although the adsorption is mainly based on weak reversible interactions, the hydrophobic effect strengthens the progressive accumulation of dissolved molecules onto the already existing adsorbed humic layers (Piccolo 2002; Piccolo et al. 2018a). The separation from the aqueous

environment thus increases the thermodynamic barrier required for the biological oxidation and decomposition of organic components. The chemical affinity with dissolved polar organic molecules in soil solution drives their progressive incorporation into a multilayer superstructure composed by contiguous hydrophilic/hydrophobic domains in the close proximity of soil aggregates. Hence, the SOC accumulation and long-term persistence are accounted to the chemical protection that excludes water and thus preserves the organic layers by microbial activity (Piccolo 2016; Masoom et al. 2016; Piccolo et al. 2018a). The wet and drying cycles of soil aggregates further contribute to separate hydrophobic compounds from the soil solution and soil microbes, thereby increasing the mean residence time of Humeome components. This organic barrier may hence provide a biochemical hindrance to microbial decomposition and develop a dynamic mechanism based on the hydrophobic protection of biolabile organic compounds (Spaccini et al. 2002a). This process thus promotes the stable incorporation of potential bioavailable compounds, such as polysaccharides, peptides, P-containing compounds, deriving from either OM inputs, crop residues, roots exudation or microbial biomass (Piccolo 2012, 2016; Piccolo et al. 2018a).

The role of hydrophobic protection in the processes of SOC incorporation and stabilization has been tested in dedicated laboratory experiments. In a short-term incubation test, a ^{13}C -labelled 2-decanol was added to soil either alone or in combination with solutions of two different humic acids (HA) from lignite and compost. At defined sampling periods, the residual amount of labile alcohol was carefully measured in both bulk samples and soil size fractions by GC-IRMS (isotopic ratio mass spectrometry) analyses (Spaccini et al. 2002b). Results revealed a rapid loss of labelled carbon in control test with the biolabile 2-decanol alone, while a continuous preservation of ^{13}C -OC was found in soil samples added with exogenous HA. The larger the hydrophobic character of HA, the slower and smaller was the mineralization of the ^{13}C -OC. It was pointed out that while the unprotected bioavailable alcohol underwent a fast microbial decomposition, the partition in the hydrophobic domains of humic matter reduced the mineralization of the labelled compound. Moreover, at the last sampling time, the largest amount of residual labelled alcohol was found in soil that had received HA additions, thereby confirming the effective incorporation and preservation of labile component in the soil Humeome (Spaccini et al. 2002b).

These findings were further evaluated in one-year long experiment, where two soils with contrasting textural composition were treated with mature compost (Cmp), humic acids isolated from compost (HA-C) and lignite (HA-L), and a polysaccharide gum (AG). Compost and humic fractions were added to soils either before or after amendment with two different doses of polysaccharidic gum, in order to verify the effect of different humified materials on the mineralization of the biolabile gum (Piccolo et al. 2004). The variation in soil TOC content was monitored monthly for each soil treatment. Notwithstanding the comparable initial TOC content, an effective stabilization of labile OC was found, for both soil types treated with bulk compost and HA-C and HA-L mixed with polysaccharides, irrespective of the timing and doses of the AG additions. As compared to control samples that only received the labile AG alone, the mixing with humified materials revealed a progressive decrease in OC losses at increasing incubation time, with a final larger OC preservation in

the finer textured silty loamy soil (Piccolo et al. 2004). These results confirm that humic materials may protect from biodegradation the biolabile molecules in soil by incorporation into the stable hydrophobic domains of the Humeome.

The findings of these laboratory experiments allowed to identify the processes involved in SOC accumulation. However, the relation between the molecular recalcitrance of the Humeome and SOC stabilization was also validated in following the OM transformation during composting and SOM dynamics in fields. In fact, the increase in biochemical stability associated with the humification stage of mature composts corresponded to the progressive preservation of hydrophobic molecules (Spaccini and Piccolo 2007, 2009; Martinez-Balmori et al. 2014). Moreover, the incorporation of apolar lipid and lignin plant derivatives was found to be associated with the increase in OC content in both tropical and temperate forest and agroecosystems, under long-term SOM management practices (Spaccini et al. 2000, 2009, 2013; Zhou et al. 2010; Song et al. 2013; Scotti et al. 2016).

Similar indications were found in long-term experiments conducted on weathered soils of Ethiopia and Nigeria, where SOC dynamics were evaluated following the vegetation shift from forest to cultivated soils. In these experiments, the soil TOC content, the NMR spectra of humic substances extracted from treated soils and the changes in ^{13}C -OC-labelled SOM due to replacement of C3 natural vegetation with C4 crops were followed (Piccolo et al. 2005a; Spaccini et al. 2006). In such highly weathered tropical conditions, despite the incorporation of recent C4 plant residues, 5 years of intensive cropping determined a decrease in SOC content in cultivated plots ranging from 46 to the 75% of the original forest soils. The molecular characterization of humic substances showed a clear relationship between the decrease in SOC content and the progressive lowering of hydrophobicity of the Humeome in cultivated plots. The authors pointed out that the reduced OM incorporation and increased mineralization of biolabile compounds were caused by the progressive loss of protective properties by hydrophobic compounds which were originally accumulated in soil under the pristine forests.

The SOC hydrophobic protection by the Humeome was verified in agricultural soils of temperate Mediterranean region for maize cropping systems (Spaccini and Piccolo 2012). In a 3-year experiment at three different field sites, the soil amendment with two annual rates of humified mature compost (2.7 and 5.4 OC ha⁻¹) was compared with conventional SOM management represented by minimum tillage and green manuring in soils undergone common ploughing and mineral fertilization. After 4 years, the amount of TOC in both bulk soils and water-stable aggregates of ploughed horizons (0–30 cm) did not reveal any significant difference between control and soils under green manuring. The soil treatment with minimum tillage showed annual and site-specific response with short-term and even negative effect on OC accumulation. Conversely, the soil amendment with hydrophobic mature composts produced a significant stabilization of SOM, revealing a persistent incorporation of SOC in both bulk samples and soil aggregates. Depending on the soil bulk density of each experimental site, field plots added with compost maintained a significant amount of added OC with compost, ranging from the 52 to 63% and from the 50 to 80%, for the low and high doses of amended compost, respectively.

These figures corresponded to a stable OC incorporation of 2.1–4.1 Mg ha⁻¹ y⁻¹ besides the OC added with compost. Moreover, ¹³C CPMAS NMR spectral data on humus extracted from the treated soils, combined with multivariate analyses, revealed that compost-amended soils progressively incorporated alkyl and aromatic hydrophobic components into the soil Humeome during the experimental time (Spaccini and Piccolo 2012). These findings further highlight that the humification in soil proceeds by an increasing accumulation of less decomposable hydrophobic material that enables the progressive entrapment and protection of the biolabile compounds exuded by root or released by degrading crop residues.

This concept of humification has been recently adopted to develop an innovative model depiction of SOC dynamics, based on the effect of molecular recalcitrance of soil Humeome components on both SOC incorporation and physical aggregation mechanisms (Mazzoleni et al. 2012). The conceptual framework of the soil organic matter dynamics (SOMDY) model combines the molecular characterization of SOM and of exogenous OM inputs, as determined by ¹³C CPMAS NMR, with their association with soil mineral particles. The vector descriptors of OC dynamics accurately anticipate both the decomposition/stabilization behaviours of a wide range of OM inputs and their effect on the soil aggregation processes (Incerti et al. 2017; Sarker et al. 2018). The close relationship between measured and simulated data obtained by the model confirmed that the molecular composition of the soil Humeome plays an important role in SOC stabilization and aggregation processes, being more predictive of SOM dynamics than the classical but less specific elemental features (e.g. C/N, lignin/N ratios) used in the current modelling approaches.

7.3.4 *Effect on Inorganic and Organic Pollutants in Soils*

The Humeome is an important environmental “buffer” that controls the fate of pollutants in soils, sediments and aqueous systems (Kozak 1996; Sannino and Piccolo 2013). The interactions promoted by humic fractions strongly determine the mobility and the bioavailability of xenobiotic compounds (Van Lipczynska-Kochany 2018). In fact, the molecular features and flexible conformational properties of humic superstructures are at the basis of a number of physical–chemical reactions with inorganic and organic chemicals.

The polar functional groups of humic molecules can complex the soluble and exchangeable forms of heavy metals, decrease their adsorption on mineral surface and counteract their negative effect on soil biological properties (Piccolo 1989; Zheng et al. 2018). Moreover, the formation of metal–humic complexes modifies the temporary bioavailability of less soluble heavy metals, thus preventing their transformation into insoluble species and improving the potential removal of these metal species (Halim et al. 2003).

The Humeome may also affect the adsorption, retention and biodegradation processes of organic pollutants. The same weak forces that stabilize the humic supramolecular conformations facilitate the incorporation of exogenous xenobiotic

materials. The various organic pollutants may bind to humic molecules through several mechanisms, including hydrogen bonding, van der Waals forces, charge transfer and hydrophobic π - π bonds (Schwarzenbach et al. 2003b). The chemical affinity with humic molecules and the poorly soluble organic pollutants fosters their selective incorporation and subsequent retention in the hydrophobic domains of the Humeome superstructures.

These thermodynamic processes driving the humic-pollutants interactions have been clearly highlighted by specific studies with NMR spectroscopy (Šmejkalová and Piccolo 2008a, b; Šmejkalová et al. 2009). NMR measurements by spin-lattice (T1) and spin-spin (T2) relaxation times and diffusion-ordered spectroscopy (DOSY) were applied to investigate the association of 2,4-dichlorophenol (DCP) with fulvic acid and two humic acids of different conformational structure. Results indicated that the larger the hydrophobic character of dissolved humic substances, the greater was the non-covalent binding of the pollutant. In fact, DCP became increasingly associated in host-guest complexes with the stable hydrophobic domains of dissolved humic matter. Moreover, the chemical shift variations for the three DCP aromatic protons indicated π - π interactions, rather than H-bonding, as the main driving force for non-covalent association between DCP and dissolved humic substances (Šmejkalová and Piccolo 2008a, b).

By similar NMR approaches, the effect of different chemical structures of organic pollutants on the interaction with HS was followed. Non-substituted (phenol P) and halogenated phenols (2,4-dichlorophenol (DCP); 2,4,6-trichlorophenol (TCP); and 2,4,6-trifluorophenol (TFP)) were solubilized in the presence of different concentration of dissolved humic acid (Smejkalova et al. 2009). The progressive decrease in molecular mobility of dissolved pollutants at increasing HA concentration indicated the formation of stable non-covalent interactions. Binding increased by lowering solution pH value, thus revealing a dependence on the protonated forms of phenols in solution. However, it was found that the hydrophobicity conferred to phenols by chlorine atoms on aromatic rings was a determinant variable for the repartition within the HA hydrophobic domains. Calculated values of Gibbs free energy of transfer and related binding constants (K_a) revealed that the more hydrophobic and less soluble the chlorinated phenols, the more extensive was the binding to humic fractions, even at low HA concentration.

Also, the hydrophilic fraction of the Humeome becomes responsible for the associations with water-soluble organic pollutants. The association of glyphosate (N-phosphonomethylglycine) herbicide (GLY) with soluble fulvic acids (FA) and humic acids (HA) at different pH values was studied by ^1H - and ^{31}P -NMR spectroscopy (Mazzei and Piccolo 2012). Both the progressive broadening of GLY signals and the modification of NMR relaxation times with ever larger humic concentration indicated the occurrence of weak non-covalent interactions between GLY and humic molecules. Spectral data obtained at different pH values (5.2 and 7) showed a stronger binding with hydrophilic FA than with more non-polar HA and more extensively at pH value of 5.2 than at pH value of 7, thereby suggesting formation of hydrogen bonds between the carboxyl and phosphonate groups of GLY and the protonated oxygen functions in humic matter. The careful measurement of association con-

stants (K_a) and Gibbs free energies of transfer obtained by diffusion-ordered NMR spectroscopy confirmed the thermodynamically favoured non-covalent association of GLY with the functional groups of FAs at pH 5.2 (Mazzei and Piccolo 2012).

The range of physical–chemical interactions between the Humeome and various pollutants suggests that humic substances may play an innovative eco-friendly role in developing a sustainable management of polluted soils (Sannino and Piccolo 2013). This may go as far as counteracting the effect of toxic soil pollutants on plant growth. A recent work combined the application of humic acid with arbuscular mycorrhizal fungi (AMF) in a Hg-polluted sandy soil cultivated with lettuce in order to verify the effects on the Hg tolerance of lettuce plants (Cozzolino et al. 2016b). It was found that plant biomass was significantly increased by the combined effect of AMF and humic acid treatments. Addition of humic matter to soil effectively complexed Hg and thus favoured the AMF capacity to improve plant nutrition, by enhancing the pigment content in plant leaves and inhibiting both Hg uptake and Hg translocation from roots to shoots.

Water-soluble humic substances may act as cheap and eco-friendly alternative to natural surfactants in the removal of inorganic and organic xenobiotics from soil through the washing technology. In fact, the use of synthetic surfactants in cleaning polluted sites by soil washing should be discouraged due to their toxicity to the soil biological quality, whose reduction will prevent any further natural attenuation of pollutants. Conversely, dissolved humic substances are natural surfactants without bearing any toxicity to soil microbial biomass. Moreover, the heterogeneous molecular composition of the Humeome enables a simultaneous removal of different organic and inorganic pollutants, thereby simplifying the soil washing steps and concomitantly enriching the soil with carbon useful to microbial metabolic processes.

Humic substances were successfully applied to remove polychlorinated biphenyls from model soils (Fava and Piccolo 2002), to reduce sorption of organic pollutants on spiked soils, thereby enabling desorption-remediation processes of polyaromatic hydrocarbons (PAHs) (Conte et al. 2001) and heavy metals (Halim et al. 2003). A water-soluble HA from leonardite was used for the soil washing of two highly polluted soils from the Italian polluted site of the ACNA industry, and its removal efficiency was compared to that of two classical synthetic surfactants, sodium dodecylsulphate (SDS) and Triton X-100 (TX100) (Conte et al. 2005). The soil washing with the HA solution showed even a slightly greater removal of a wide range of organic pollutants (PAHs, monoaromatic halogenated and nitrogenated compounds, thiophenes, sulphones, biphenyls) than by conventional surfactants, thereby proving the large surfactant activity of humic matter. An advantage over the synthetic chemicals is that humic materials are natural organic compounds, which do not exert toxicity on the microbial biomass and may instead stimulate the biological properties of polluted soils and promote a subsequent biological attenuation and a restoration of the biological quality of cleaned soils.

7.4 Case Studies

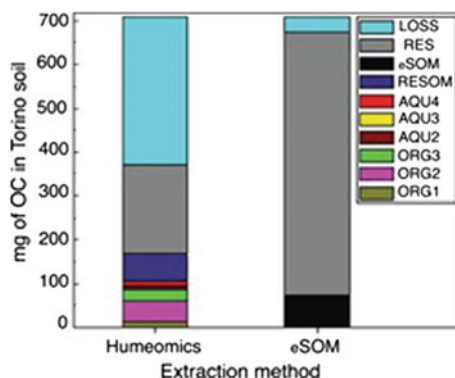
7.4.1 Direct Application of Humeomics on Soils

The application of the Humeomics fractionation on humic matter has allowed significant advancements in understanding HS molecular composition. However, an ideal SOM fractionation method should also be applicable directly on soils. Hence, Drosos et al. (2017) recently subjected an agricultural sandy loam to a complete Humeomic fractionation protocol. The residual OM still present in soil after the sequential fractionation was removed by an alkaline extraction providing a final fraction referred to as RESOM. Concomitantly, SOM was extracted by the traditional method employing a solution of NaOH and $\text{Na}_4\text{P}_2\text{O}_7$ and referred to as eSOM. The humic molecules released in each fraction were characterized by NMR, GC-MS and LC ESI-Orbitrap-MS.

The unbound fraction (ORG1) showed a larger amount of long alkyl chains than the other fractions, mainly unsaturated, mono- or poly-hydroxylated compounds. The ORG2 fraction contained fatty acids and sugars, and a minority of phenolic acids, imines, esters, dicarboxylic acids and hydroxyacids. Conversely, most molecules in the AQU2 fraction were ascribed to *O*-alkyl-containing compounds, with a small amount of alkyl and aromatic moieties. Other AQU fractions characterized by Orbitrap-ESI-MS showed the presence of amides, benzoic acids, nitrogen and oxygen heterocyclic compounds, ethers and a minority of phenolic esters, phenolic acids, hydroxyl acids, sterols, esters and alcohols. Furthermore, evidence of the occurrence of adducts between water-soluble humic components and Fe ions was also observed. The complexes were taken into account by further numerical elaboration of the empirical formulae obtained by MS and found that one to three Fe ions were covalently bound to humic molecules (Drosos et al. 2017). The molecular composition of the different separates by Humeomics was found similar to that reported for humic extracts, except for a greater abundance of cyclic acids (Nebbioso and Piccolo 2012).

The molecular composition of the RESOM fraction was compared to that of the eSOM extract. The smaller content of *O*-alkyl carbons in NMR solid-state spectra of RESOM than in those of eSOM was taken as an evidence of the progressive removal of hydroxyl-containing compounds (e.g. alcohols, saccharides) during Humeomics fractionation. Conversely, the intense alkyl peaks still detected in NMR spectra of RESOM indicated a persistent content of alkyl compounds tightly bound to soil minerals. This corroborates the fundamental role of hydrophobic components in stabilizing the conformation of humic suprastructures and in favouring the accumulation of organic carbon in soil (Piccolo 2001, 2012; Nebbioso et al. 2014a). In fact, the recalcitrance of hydrophobic compounds to the extraction even by Humeomics indicates an ultimate chemical inaccessibility that is likely due to their intimate interaction with soil mineral components. These findings show that RESOM stabilization should be attributed to the adsorption of its hydrophobic components on the alumina-silicate

Fig. 7.3 Organic carbon (OC) yields of Humeomic fractions (ORG1-3, AQU2-4 and RESOM) versus eSOM, including the OC of the unextractable material in both cases (RES) and of the material lost after extraction (LOSS)



surfaces and to the formation of complexes between oxygen-containing hydrophilic groups and mineral iron.

Drosos et al. (2017) have also reported the mass yield (mg) of organic C (OC) obtained by either the traditional alkaline extraction (eSOM) or the Humeomics fractionation (Fig. 7.3). The traditional alkaline extraction was found significantly less efficient than the Humeomics in isolating humic compounds from soil. In fact, the authors pointed out a solubilization of 66% of the soil Humeome by Humeomics that was instead unextractable and, thus, unidentifiable by the traditional alkaline method (Drosos et al. 2017). Even though the C lost during eSOM extraction was smaller than for Humeomics, the unextractable OM in the case of eSOM was significantly greater (Fig. 7.3). The authors ascribed such a different solubilization of humic matter to the larger capacity of Humeomics to access humic molecules, which were progressively unmasked by first removing unbound organosoluble compounds and then the weakly and strongly bound organosoluble (ORG2, ORG3) and hydrosoluble (AQU2, AQU3) molecules after hydrolyses of esters of increasing stability, and, finally, the water-soluble AQU4 fraction released after breaking ether bonds.

This work highlighted the potential of Humeomics in unravelling the molecular nature of an amount of soil humic molecules far larger than that usually reached by traditional alkaline extraction of SOM and showed for the first time the molecular role of Fe in promoting the stabilization of OM in soil (Drosos et al. 2017). Moreover, the reported high abundance of amines, amides and heterocyclic N-compounds, especially in AQU fractions, may contribute to shed light in the role of organic N-containing molecules in the reactivity of Humeome in soils and towards biota. Finally, results of Drosos et al. (2017) confirmed that the high-resolution Orbitrap-MS was more helpful in characterizing humic molecules than other analytical tools, such as low-resolution GC-MS or the poorly sensitive NMR spectroscopy (Saiz-Jimenez 1995; Šmejkalová et al. 2008a, b).

Humeomics was also exploited to assess and compare the molecular composition of the Humeome of two different grassland soils (Soil A and B) (Drosos et al. 2018b). All Humeomic organic and aqueous fractions, as well as RESOM and eSOM, were studied by HPSEC hyphenated to ESI-Orbitrap-MS. The spectrograms provided 175

empirical formulae for Soil A and 139 for Soil B, which were discussed in relation to their molecular size, unsaturation degree, oxygenation and the presence of N in the formula. Compounds containing only one N atom and showing no or low unsaturation were likely of microbial origin, while highly oxygenated molecules containing or not containing N atoms were mainly ascribed to saccharide moieties. Due to a predominant anoxicity (depletion of dissolved oxygen), Soil B contained a larger amount of nitrogenated compounds in the Humeome and a lower C/N ratio than Soil A. Conversely, the more oxic Soil A contained more highly oxygenated and unsaturated molecules than Soil B (Drosos et al. 2018b).

The observed empirical formulae were matched with their molecular structures, making use of ChemSpider and PubChem databases. Hence, the observed molecules were distributed into 16 specific chemical groups, whose distribution in the two soils was visually shown by Van Krevelen plots. By this approach, the organosoluble fractions in both soils were found to be dominated by aliphatic amides and saccharide ethers, while the hydrosoluble fractions comprised mainly aromatic amides, heterocyclic nitrogen compounds and saccharide ethers. Moreover, 66 molecules had been found common in both soils, with a main abundance of saccharide ethers, which were additionally bound to aromatic compounds in the water-soluble fractions. The study not only corroborated the potential of Humeomics in the clarification of the structure of N-containing compounds, but also highlighted the role of Humeomics in clarifying the mechanisms of long-term persistence of humic molecules in soil (Drosos et al. 2018b).

A similar Humeomic approach was adopted by Drosos and Piccolo (2018) to study the molecular features of the Humeome of a sandy loam agricultural soil that was cultivated with maize. Soil samples were collected after one and three years of maize cultivation under continuous conventional tillage and subjected to both the alkaline extraction and the Humeomic fractionation. As elsewhere, the extracts were characterized by GC-MS, HPSEC-ESI-Orbitrap-MS and elemental analysis. Twenty-six different classes of compounds were characterized by Humeomics, while only 11 classes were found in eSOM. In particular, 53 and 31 molecules were identified in eSOM of the first and third tillage year, whereas Humeomics allowed the structural assignment of 353 different molecules in the first year and 366 different molecules in the third year. While SOC extracted in eSOM was about 14.0% of total SOC in both years, the full Humeomic procedure allowed to extract 50.8% of total SOC after the first year (i.e. 3.65 times more C than for eSOM) (Drosos and Piccolo 2018). Furthermore, the Humeomic fractionation revealed both a progressive loss of hydrophobic protection in soil and a concomitant increase in SOM decomposition, due to a smaller ratio of organosoluble to hydrosoluble OC (from 0.77 to 0.59), passing from the first to the third year.

It was also found that several heterocyclic N-containing molecules constituted the main compound class in both eSOM and RESOM for both tillage years. Such molecules could have possibly derived from the methoxylation of tryptophan bound to iron hydroxide in soil (Nuzzo et al. 2013). Interestingly, most amides were found in both the first and third year Humeomes, even though their amount was reduced, thereby suggesting that such molecules have been lost with tillage. Conversely, the

amines showed a greater resistance to tillage-induced transformation, since about the 99% of such compounds have been identified in the soil Humeome after both cultivation years (Drosos and Piccolo 2018). The overall recalcitrance of N-containing structures to soil tillage has been explained by the authors with the formation of covalent bonds between N and Fe in iron hydroxides. The Fe-bound molecules found in eSOM extracts were only slightly decreased from first to third year of tillage, whereas total Humeomics not only enabled the isolation of a larger number of these structures, but also revealed that their amount increased with tillage (Drosos and Piccolo 2018). Moreover, the quantitative increase from first to third year samples was 6% for heterocyclic N-containing molecules, 21% for amine and even 71% for phenolic ester. These values were in line with those reported for model systems based on Fe complexes with amines and heterocyclic N-containing compounds (Orlowska et al. 2017).

The Humeomic fractionation of the maize-cultivated soil also enabled to find an explanation for the results by Grignani et al. (2012), who reported that the biomass yields for maize grown on the soil fractionated by Humeomics by Drosos et al. (2018a, b) were reduced in the third year of tillage, more than in the first year. In fact, while the fertility loss could not be appreciated by changes in eSOM, Humeomics allowed to possibly relating this loss to a decrease in the overall aliphatic moieties of SOM (Drosos and Piccolo 2018). A decrease of hydrophobic carbon in soil is known to weaken the stability of soil aggregates, with a consequent decrease in the general soil quality (Piccolo and Mbagwu 1999; Piccolo et al. 2018a).

The advantage of the direct Humeomics application on soil is multiple. First of all, the fractionation of the soil Humeome is in itself a confirmation of the supramolecular nature of soil organic molecules, since they are progressively released from domains in which polar compounds (AQU fractions) are surrounded by or contiguous to hydrophobic structures (ORG fractions). These mixed hydrophilic/hydrophobic humic domains are not only stabilized by hydrogen, π - π and metal-bridged bonds, but also by covalent bonds with either Fe or Al-Si components of oxides, hydroxides and clay minerals, thereby forming stable organo-mineral associations (Drosos et al. 2017). Moreover, Humeomics allows the characterization of more hydrophobic components of the Humeome, due to the lipid-rich ORG fractions, which are not solubilized in eSOM. The fact that most of ORG molecules are common among the ORG fractions becomes a further evidence of the supramolecular arrangement of the soil Humeome (Piccolo 2001; Piccolo et al. 2018a).

These considerations suggest that Humeomics, due to its capacity to show the changes in SOM composition with time or different experimental conditions, represents the most advanced tool for the ultimate assessment not only of the molecular composition of the soil Humeome, but also of its dynamics. For example, the molecular information provided by Humeomics may be used to introduce an index related to C stabilization in soil. In fact, since the lipidic compounds are thought to protect hydrophilic components from mineralization and microbial degradation (Piccolo et al. 2004; Spaccini et al. 2002b), the larger the organosoluble/hydrosoluble carbon ratio of the soil Humeome, the greater is the chemical protection of organic matter in

a soil. Therefore, in the case of the agricultural soil studied by Drosos et al. (2017), a chemical protection ratio (CPR) has been developed (Eq. 7.1, Piccolo et al. 2018a).

$$\text{Extractable OM Chemical Protection Ratio (CPR)} : \frac{\sum \text{ORG}_i \text{OC}_{\text{mg}}}{\text{RESOM OC}_{\text{mg}} + \sum \text{AQU}_i \text{OC}_{\text{mg}}} \quad (7.1)$$

This CPR ratio based on the OC in ORG, AQU and RESOM fractions separated by Humeomics may be useful to evaluate the efficacy on SOM content of different soil management aimed to sequester C in soil. In fact, in the case of the work by Drosos and Piccolo (2018), an enhancement of the CPR value above 1.05 signified that the soil reached a more chemically protected status under a particular soil treatment. Conversely, a decrease in the CPR ratio would suggest a tendency of the soil to an advanced mineralization of SOC. It may be envisaged that similar alterations of the soil Humeome may be shown as a function of soil type, soil cropping and tillage systems. In fact, such management practices may affect the supramolecular structure of the soil Humeome and consequently alter its dynamics. Therefore, within the intensification of soil exploitation expected in the next decades with population growth, it becomes essential to rely on an advanced technique, such as Humeomics, that may efficiently provide insights into the relationship between the structure of the complex and heterogeneous soil Humeome and its functional reactivity in the environment (Piccolo et al. 2018a).

7.4.2 *Carbon Sequestration by the in Situ Photocatalytic Polymerization of the Soil Humeome*

The current view depicts the soil Humeome as supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces. This novel paradigm of the chemical nature of soil humus allows conceiving innovative methodologies capable of promoting an effective SOC sequestration based on the inherent molecular characteristics of the soil Humeome. In this respect, an example of an innovative sustainable soil management towards an increased sequestration of SOC is represented by the implementation of a chemical technology aimed to bind together the different humic molecules by covalent bonds in larger oligomers and consequently increase the average molecular mass of the soil Humeome (Piccolo 2012; Piccolo et al. 2018a, b). This structural modification is hence expected to increase the internal energy of the Humeome and reduce or even inhibit the microbial mineralization of SOM.

The addition to solutions of humic molecules of an oxidative enzyme such as horseradish peroxidase, together with an oxidant such as hydrogen peroxide, induced a free radical-driven coupling reaction among humic aromatic molecules, with an effective oligomerization of the humic matter (Piccolo et al. 2000; Cozzolino and Piccolo 2002). A technological improvement was obtained by replacing the bi-labile enzyme with synthetic *biomimetic* catalysts such as metal porphyrins that

mimic the activity of oxidative enzymes. To ensure water solubility and stable environmental reactivity, the porphyrin ring was chemically modified, by including electron-withdrawing dichloro-sulphonatophenyl groups. These water-soluble metal porphyrins were found to easily catalyse, under H_2O_2 oxidation, the covalent coupling of humic phenol components in greater molecular mass molecules (Piccolo et al. 2005b; Šmejkalová and Piccolo 2006; Šmejkalová et al. 2006).

Even more interesting was the finding that the formation of intermolecular C–C and C–O–C bonds among humic molecules catalysed by metal porphyrins could be equally achieved under photo-oxidation by simply exposing the reactive components to solar irradiation. The in situ catalysed photo-polymerization of SOM was shown in a laboratory experiment, whereby soils treated with iron porphyrin under solar light were found not only to significantly increase soil physical stability despite several cycles of wetting and drying but also to reduce microbial respiration and CO_2 emission to the atmosphere (Piccolo et al. 2011). Similar findings were shown by a mesocosm experiment with undisturbed soils treated with iron porphyrin and exposed to solar light (Gelsomino et al. 2010). These results indicated that the catalysed photo-polymerization of SOM enabled both an enhanced soil aggregation due to more extensive soil particles interactions and a reduced capacity of soil microorganisms not only to reach the larger humic molecules adsorbed on soil particles but also to overcome the increased internal energy of the newly formed covalent bonds.

The efficacy of the biomimetic catalysis on the photo-polymerization of humic molecules was further increased when a manganese porphyrin was immobilized through an imidazole spacer to the surface of common clay minerals such as kaolinite and montmorillonite (Nuzzo and Piccolo 2013; Nuzzo et al. 2013). The metal porphyrin immobilization on eco-compatible solid surfaces entailed a heterogeneous catalysis three times more efficient than the homogeneous catalysis by a water-soluble metal porphyrin. The enhanced efficiency of the heterogeneous catalysis was attributed to the larger persistence of the immobilized biomimetic catalyst on the soil surface, whereas the water-soluble metal porphyrins are easily eluviated down the soil profile, thus losing the capacity to benefit from the photo-oxidation that occurs at the soil surface (Nuzzo et al. 2016, 2017).

These positive findings were applied in real field conditions within an Italian national research project (MESCOSAGR) focused on the development of sustainable SOC sequestration methods in agricultural soils. The water-soluble iron porphyrin catalyst was amended to field soils under wheat cultivation at three sites along a north–south climate gradient in Italy (Spaccini and Piccolo 2012). After three years, the photo-polymerization of SOM promoted by the biomimetic catalyst increased the average TOC content in both bulk soils and their size separates from 2.24 to 3.90 $\text{t ha}^{-1} \text{y}^{-1}$ in respect of control plots (Piccolo et al. 2018b). This exceptional extent of soil carbon sequestration was much larger than what is currently achieved by no-tillage practices which are reported to hardly exceed 0.5 $\text{tC ha}^{-1} \text{y}^{-1}$ (Minasny et al. 2017). Since the experimental field plots were subjected to a conventional tillage system without any additional OM inputs, this work showed that the in situ catalysed photo-oxidative treatment effectively enhanced the chemical and biochemical stability of

the soil Humeome, thus favouring a progressive SOC sequestration. Moreover, the objectively considerable amount of carbon sequestered yearly in this study by the applied photocatalytic technology was even achieved without either altering the soil biological quality or reducing the crop yields (Piccolo et al. 2018b). Fixation of SOC by amending soils with a photocatalyst, such as metal porphyrins, may be promising to establish an easy soil management practice that not only controls SOM dynamics and contributes to accumulate carbon stocks in agricultural soils, but also sustains the intensification of agriculture required to feed an increasing population.

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