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A molecular zoom into soil Humeome by a direct sequential chemical fractionation of soil

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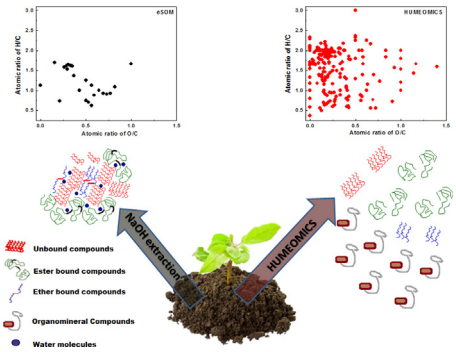
HIGHLIGHTS

- Soil Humeome was for the first time directly fractionated from soil.
- 235% more carbon extracted by Humeomics than traditional alkaline extraction.
- Separation of soil Humeome unraveled soil organominerals arrangement.
- HR-ESI-Orbitrap-MS revealed humic molecules covalently bound to Fe and AlSi.
- 66% of unextractable carbon by alkaline solution was solubilised by Humeomics.

GRAPHICAL ABSTRACT



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ABSTRACT

A Humeomics sequential chemical fractionation coupled to advanced analytical identification was applied directly to soil for the first time. Humeomics extracted ~235% more soil organic carbon (SOC) than by the total alkaline extraction traditionally employed to solubilise soil humic molecules (soil Humeome). Seven fractions of either hydro- or organo-soluble components and a final unextractable humic residue were separated from soil. These materials enabled an unprecedented structural identification of solubilised heterogeneous humic molecules by combining NMR, GC–MS, and ESI-Orbitrap-MS. Identified molecules and their relative abundance were used to build up structure-based van Krevelen plots to show the specific contribution of each fraction to SOC. The stepwise isolation of mostly hydrophobic and unsaturated molecules of progressive structural complexity suggests that humic suprastructures in soil are arranged in multi-molecular layers. These comprised molecules either hydrophobically adsorbed on soil aluminosilicate surfaces in less stable fractions, or covalently bound in amorphous organo-iron complexes in more recalcitrant fractions. Moreover, most lipid molecules of the soil Humeome appeared to derive from plant polyesters rather than bacterial metabolism. An advanced understanding of soil humic molecular composition by Humeomics may enable control of the bio-organic dynamics and reactivity in soil.

1. Introduction

Soil contains the largest terrestrial pool of organic carbon (OC), with global estimates ranging from 1115 to 2200 Pg (Batjes, 2014). Recent evaluations of SOC dynamics have shown that 20–40% of carbon stored as organic matter (OM) in the upper soil layers has turnover times

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of centuries or less. This fast-cycling OM largely comprises undecomposed plant material and hydrolysable components weakly stabilized in association with mineral surfaces (Trumbore, 1997). On the other hand, the persistent humic carbon in passive soil pools has a much longer turnover time depending on soil age and mineralogy (Sollins et al., 1996). Although, at a global scale, the soil carbon pool is 3.3 and 4.5 times larger than the size of the atmospheric and biotic pools (Lal, 2004), respectively, the dynamics of soil humus remain still poorly understood after nearly a century of study (Trumbore, 1997), due to the multiplicity of factors that affect SOC stabilization. Industrial agricultural practices accelerate the decline of humus content in soil, and, consequently, the reduction of soil fertility, biodiversity, and soil structural stability (Reeves, 1997; Fontaine et al., 2007), while enhancing greenhouse gases emissions from soil (Smith et al., 2014). Since it is the specific molecular composition of the soil Humeome (Piccolo, 2002) that significantly affects SOC storage dynamics (Woo et al., 2014), soil basal respiration (Fang et al., 2005), and humus-plant relationships (Kelleher and Simpson, 2006; Canellas and Olivares, 2014), it becomes necessary to reach a rigorous identification of the molecular structure of SOC components (Hedges et al., 2000; Schmidt et al., 2011).

A novel understanding of the soil Humeome has recently emerged from experimental evidence. Rather than being composed of large molecular weight macropolymers, as traditionally believed, Soil Organic Matter (SOM) is now regarded as a non-covalent supramolecular association of small heterogeneous molecules, that survive microbial degradation of plant and animal tissues, and are held together mainly by weak dispersive forces (Piccolo, 2001, 2002). It is acknowledged that the extraordinary molecular complexity of SOM comprises the multiple metabolic products of plants degradation and soil microbial activities, which are stabilized in the three-dimensional inorganic soil matrix (Schmidt et al., 2011).

Alkaline extraction is traditionally used to isolate humic molecules from soil (Stevenson, 1994). However, the greatly complex molecular fragments obtained are often believed not to represent the original arrangement of SOM (Schmidt et al., 2011; Lehmann and Kleber, 2015). Moreover, minerals like Fe and Al can aggregate humic molecules in a carbon pool that may not be extractable by the alkaline extraction method (Jansen et al., 2011). To reduce the heterogeneity of SOM molecules and facilitate their characterization, humic extracts (Humic Acid, and Fulvic Acid) isolated from soil by alkaline dissolution were subjected to a number of fractionation procedures, based on either polarity or molecular size of fractions (Otsyki and Hanya, 1966; Wershaw and Pinckney, 1973; Curtis et al., 1981; Brown et al., 1999; Tombacz, 1999; Christl et al., 2000; Barber et al., 2001; Conte et al., 2006; Leenheer, 2009; Li et al., 2009; van Schaik et al., 2010; Canellas et al., 2010; Drosos et al., 2014). However, an ideal SOM fractionation method should be directly applied *in situ* to soil, designed to reduce molecular alterations, and maximize the number of observable molecules (Lehmann and Kleber, 2015). A mild sequential chemical fractionation from the humic supramolecular matrix, named Humeomics, was recently developed (Nebbioso and Piccolo, 2011, 2012; Nebbioso et al., 2014a, 2014b, 2015) to avoid the deliberate breaking of any C—C bond during separation. An unbound fraction extracted with organic solvent was followed first by a weakly-bound and then a strongly-bound fraction solvated by a boron trifluoride transesterification, and a methanolic alkaline hydrolysis, respectively, and by a final cleavage of ether and glycosidic bonds with hydroiodic acid. The humic molecules released in each separate fraction were characterized in detail by advanced analytical instrumentation.

Here, we applied Humeomics for the first time directly on soil, with the aim to isolate more homogeneous SOM molecular components than by the traditional alkaline extraction and reach a more comprehensive understanding of their structure, as well as their conformational arrangement in soil.

2. Experimental

2.1. Extractable SOM in an alkaline solution (eSOM)

Triplicates of 100 g of an agricultural sandy loam soil sampled near Torino, Italy, and classified as Typic Ustifluvent (Piccolo, 2012), were used to extract eSOM in 0.9 L of an alkaline solution (0.5 M NaOH + 0.1 M $Na_4P_2O_7$), as by traditional methods of humus extraction. After overnight shaking, eSOM was separated from soil by centrifugation (15 min, 4500 rpm), filtered through a Whatman 41 filter, and adjusted to pH 7 with 37% HCl. Then, eSOM was dialyzed against distilled water using Amicon C membrane (1000 Da cutoff) to remove residual salts and freeze-dried to collect 1250 (±50) mg of extract.

2.2. Humeomics sequential fractionation

Triplicates of 100 g of the same soil were placed in 300 mL of 0.1 M HCl and shaken overnight. The samples were centrifuged (15 min, 4500 rpm), water-washed until neutrality and air-dried prior to Humeomics separation of fractions.

2.2.1. Unbound fraction (ORG1)

ORG1 was extracted under stirring for 24 h at room temperature from 100 g of washed soil suspended in 300 mL of a 2:1 v/v dichloromethane (DCM) and methanol (MeOH) solution. The supernatant was separated by centrifugation (15 min, 7500 rpm) and filtration. The soil residue left on the filter was air-dried.

2.2.2. Weakly bound ester fractions (ORG2 and AQU2)

The residue from step 1 was placed in a Teflon tube added with 12% BF_3-MeOH (1 g of soil/1 mL of solution) and kept under N_2 atmosphere overnight at 85 °C. This transesterification was repeated twice, the supernatants were centrifuged (15 min, 7000 rpm) and combined. The resulting solution was added with water to quench the residual BF_3, rotoevaporated to remove MeOH, and extracted three times with a total of 150 mL of chloroform. The organic phase was separated (ORG2), dried with anhydrous Na_2SO_4, filtered on a Whatman 41 filter, and rotoevaporated. The aqueous phase (AQU2) was rotoevaporated to remove residual MeOH and chloroform traces, and dialyzed against distilled water using Amicon C membranes (1000 Da cutoff) until Cl-free, and freeze-dried. The remaining solid residue was air-dried before the next step.

2.2.3. Strongly bound ester fractions (ORG3 and AQU3)

The residue (1 g per mL) was suspended in 1 M KOH-MeOH solution, refluxed for 2 h at 70 °C under N_2 atmosphere. After cooling, the reaction mixture was centrifuged (10 min, 4500 rpm) and the supernatant was recovered. The residue was washed with 50 mL of MeOH and centrifuged. The supernatants were combined, their pH adjusted to 2.0 with 37% HCl, and then liquid-liquid extracted three times with total of 150 mL (50:50, v/v) of DCM/water mixture. The organosoluble (ORG3) and hydrosoluble (AQU3) extracts were purified as for ORG2 and AQU2. The solid residue was air-dried before the next step.

2.2.4. Strongly bound ether fraction (AQU4)

A suspension of 1 mL of 47% HI aqueous solution per g of soil residue was stirred for 48 h at 75 °C under N_2 atmosphere. After cooling, 100 mL of distilled water were added, stirred and filtered. The solution was neutralized by saturated $NaHCO_3$ solution, freeze-dried, and dialyzed (1000 Da cut-off membranes) first against saturated $Na_2S_2O_3$ solution to neutralize I_2, and, then, against distilled water to remove residual $Na_2S_2O_3$. The resulting suspension (AQU4) was freeze-dried. The residual soil was washed extensively with water and used to extract RESOM.

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