Drosos *et al. Chem. Biol. Technol. Agric. (2018) 5:14*
https://doi.org/10.1186/s40538-018-0127-y

Chemical and Biological Technologies in Agriculture

RESEARCH

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Structural characterization of carbon and nitrogen molecules in the Humeome of two different grassland soils

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Abstract

Background: The surface layers of two German grassland soils, a silt loam soil from Dortmund (Soil A) and a sandy loam soil from Hannover (Soil B), were subjected to the Humeomics fractionation to identify the molecular composition of the soil Humeome.

Methods: The separated Humeomic fractions were analysed by high performance size exclusion chromatography hyphenated to a high-resolution electrospray-Orbitrap Mass Spectrometry (MS). Empirical formulae were obtained by mass data to describe the carbon and nitrogen molecular structures in the soil organic matter (SOM) of both soils.

Results: Results showed 175 masses for Soil A and 139 for Soil B distributed among the Humeomic fractions. Masses were classified according to molecular weight, unsaturation degree, oxygenation, and presence of nitrogen in the formula. The molecular information obtained with MS was consistent with the physical–chemical properties and environmental condition of the two grassland soils: (i) nitrogenated compounds were generally more numerous and more relatively abundant in the Humeome of Soil B, which was more anoxic and lower in C/N ratio than Soil A, (ii) highly oxygenated compounds were more numerous and abundant in the more oxic Soil A, (iii) most unsaturated formulae were comparatively more abundant in Soil A than Soil B, in line with differences in environmental conditions. The empirical formulae were then matched with their molecular structures, based on the application of ChemSpider and PubChem databases, and were found to be distributed into 16 specific chemical groups. The Van Krevelen plots built on the resulting carbon and nitrogen molecular structures provided a visual comparison of the Humeome of the two soils. The organosoluble fractions in both soils were dominated by aliphatic amides and saccharide ethers, while the hydrosoluble fractions comprised mainly aromatic amides, heterocyclic nitrogen compounds, and saccharide ethers.

Conclusions: Even though the two soils contained different compounds, 66 molecules were found to be common. Based only on differences in soil texture and oxic conditions, these findings indicate the existence of similar molecular dynamics in the stabilization of organic matter in these two grassland soils. The main common group in the Humeome of both soils was the saccharide ethers, which were additionally bound to aromatic compounds in the hydrosoluble fractions. Our detailed molecular study of the Humeome of these grassland soils confirms the potential of the Humeomic procedure to assess not only the carbon and nitrogen molecular composition in SOM, but also the mechanism of their long-term persistence.

Keywords: Carbon, Nitrogen, Grassland soils, Molecular structure, SOM, Humic matter, Humeomics, Sequential chemical fractionation, ESI-Orbitrap-MS

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Background

Soil organic matter (SOM) plays a fundamental role in soil fertility [1]; it contributes to its physical–chemical stability [2] and interacts with heavy metals and organic hexogenous materials, including those of hazardous nature [3]. Its composition and molecular structure has baffled scientists for decades [4], although recent evidence of its supramolecular rather than macropolymeric character [5] gained a general consensus [6–10]. By this understanding, SOM contains a variety of small heterogeneous relatively small compounds which self-assemble in superstructures by weak interactions, and may thus be potentially analytically determined.

This complex supramolecular matrix was shown to have intermolecular interaction of different strength, and may only be simplified using a step-wise extraction procedure named Humeomics that involves increasingly severe extraction conditions [11]. Humeomics enabled the successful characterization of a large number of compounds [12] in purified humic substance's (HS) extracts such as humic acids (HA) [11], its size fractions [13] and humin [14]. In particular, the Humeomic procedure even improved the detection of molecular components of soil organic carbon (SOC) in the unextractable organic matter by both mass spectrometry (MS) [15] and nuclear magnetic resonance (NMR) experiments [16], and enabled the characterization of compounds rich in quaternary carbon. These findings are relevant in light of the recent interest in the characterization of highly unsaturated SOC [17–19] driven by the availability of ultra-high-resolution mass spectrometers.

Conversely, highly unsaturated soil organic nitrogen (SON) is still largely uncharacterized, even though its existence and relevance has been postulated [20]. Moreover, the role of SON in the supramolecular arrangement of the soil Humeome is still unknown, and this uncertainty is reflected in the technical difficulty of its measurement and the consequent poor knowledge on its vital plant nutrition capacity [21–23]. Further molecular understanding of the dynamics of SOC and SON would be also important for their broad ecological relevance since their recognized transport to natural waters and atmosphere [24, 25].

The presence of more types of N-containing organic constituents in soil has led to classify SON according to apparent molecular weight, chemical nature and origin. SON categories include readily extractable hydrophilic aminoacids [26] generally associated with LMW fraction of SON, while polyphenol-like matter are attributed to the HMW fraction [27]. Furthermore, heterocyclic SON has been investigated by pyrolysis [20] and later by X-ray methods [28], but both methods do not provide accurate structural information. This type of SON may be originally aromatic in nature, or possibly induced by combustion [29]. Furthermore, proteins may be another source for SON [30], which is assumed to resist degradation due to encapsulation in SOM hydrophobic domains [31], thus extending the SON lifespan [32]. Nevertheless, the molecular characterization of single components of SON has been only recently attempted and mainly focused only on DON and sediment characterization [33].

A positive correlation between fine soil texture and SON abundance was inferred [34], arguing that a physical protection is provided by finer soil particles that enable physical preservation of even the most labile N-containing compounds. Conversely, in coarser soils, molecular recalcitrance is prevalently due to a chemical mechanism, thus resulting in a greater preservation of non-nitrogenated compounds, such as lignins [34]. Furthermore, it has been shown that the presence of degradative enzymes from microbial biomass affects SOC and SON composition [35]. In a recent application of Humeomics to an Italian silt loamy agricultural soil, it was found that the main molecular components of the soil Humeome were heterocyclic nitrogen compounds (HN) and amides (AD) [36]. However, more experimental work on the stability of SON is required to confirm the importance of its heterocyclic components in soils.

While much work by Fourier transform ion cyclotron (FT-ICR)-MS is available on the characterization of environmental DOM [37] that on SOC and SON is generally lacking due to the extreme complexity of terrestrial materials [38]. Conversely, the Orbitrap-MS technique has been successfully applied for the accurate characterization of masses in humic matter and Humeomic fractions separated from soils [36, 39, 40]. In fact, despite the relatively lesser mass resolution, Orbitrap-MS can successfully enable the assignment of individually detected masses within Humeomic fractions to specific molecular formulae by improving data processing [41]. The molecular characterization of the soil Humeome rather than that of bulk SOM enables a detailed description of SOM molecular composition and lay stronger foundations for the molecular fingerprinting of soil humus [36, 42], thus representing a much more accurate alternative to current methods such as UV–Vis spectroscopy [43], MS [44], outdated degradative methods [45], and even poorly sensitive NMR spectroscopy [46]. The molecular investigation of the soil Humeome not only opens up to the correlation between SOM composition and its origin, but also enables to identify specific molecular biomarkers for different soil ecosystems, inasmuch as it is already attempted for DOM [47, 48].

The objective of this study was hence, to reach a deeper molecular knowledge of the humus composition in two

Table 1 Structural, geographical and selected physico-chemical properties of soils

	Soil A	Soil B
Origin	Dortmund Barop, An der Palmweide, Germany	Hannover Fuhrberger Feld marsh, Germany
Latitude	51.5149	52.3705
Longitude	7.4660	9.7332
Altitude (m)	96	57
Soil classification (USDA)	Haplic Cambisol	Gleyic Podzol
Clay (< 2 μm) (%)	16.00	2.40
Silt (< 50–2 μm) (%)	73.00	29.00
Sand (2000–50 μm) (%)	11.00	68.60
Textural class	Silt loam	Sandy loam
pH (water, ratio 1:2.5)	6.74	5.00
$C_{org.}$ (%)	2.88	1.88
$N_{tot.}$ (%)	0.13	0.10

Table 2 Weight yields (mg) of Humeome fractions obtained from two grassland soils and relative (%) percentage of molecules common in both soils for each Humeome fraction

Fraction	Soil A	Soil B	Relative (%) percentage of molecules common in both soils	
			Soil A	Soil B
ORG1	535	382	56.5	62.0
ORG2	1100	878	68.7	60.0
ORG3	15	46	89.7	87.2
AQU2	729	684	55.4	78.2
AQU3	9	496	24.1	85.7
AQU4	8	1	57.8	83.0
RESOM	138	321	59.5	20.4
Total Humeome	2534	2808		

different grassland soils for both its organic carbon and nitrogen forms.

Methods

Soil samples

Two surface-layered (0–10 cm) German grassland soils were collected from the Barop area in Dortmund (Soil A) and from the Fuhrberger Feld area, in Hannover (Soil B). The soils were air-dried, and sieved with a 2-mm sieve. Their characteristics are reported in Table 1.

Soil Humeome fractionation

The applied soil Humeome fractionation was applied on two replicates for each soil, as described earlier [36, 39] and briefly below. Weight yields of the separated Humeome fractions from both soils are reported in Table 2.

Unbound fraction (ORG1)

The dried soil was weighed in an amount corresponding to 1.0 g of its content in soil organic carbon (SOC) calculated from its % of OC. Soil samples were subjected to the first step by extracting the unbound organosoluble fraction (ORG1) with a dichloromethane and methanol 2:1 solution, under stirring for 8 h at room temperature. The supernatant was centrifuged (2000 rpm for 5 min), filtered through GF-C filters (Whatman), and rotoevaporated.

Weakly ester-bound fractions (ORG2 and AQU2)

The soil residue was then transferred in Teflon tubes (Nalgene) and added with 100 mL of a Boron trifluoride (Acros Organics) solution at 12% w/v in methanol and heated for 8 h at 90 °C. After cooling, the supernatant was separated and transferred in a liquid/liquid extraction funnel where addition of a 1:1 water and chloroform mixture allowed separation of the weakly ester-bound compounds in the organosoluble (ORG2) and hydrosoluble (AQU2) fractions, which were, respectively, rotoevaporated, dialysed (Spectrapore 1000 Da cut-off membranes) and freeze-dried.

Strongly ester-bound fractions (ORG3 and AQU3)

The soil residue was suspended in a KOH 1 M (Sigma-Aldrich) solution in methanol for 2 h under refluxing. The supernatant was separated and placed in a liquid/liquid extraction funnel where addition of a 1:1 water and chloroform mixture allowed separation of the strongly ester-bound compounds in the organosoluble (ORG3) and hydrosoluble (AQU3) fractions. The former was rotoevaporated while the latter was dialysed (Spectrapore 1000 Da) and freeze-dried.

Ether-bound fraction (AQU4)

Hydroiodic Acid (Acros Organics), 47% w/v in deionized water (Milli-Q) was added to the residue, heated for 72 h at 70 °C. The supernatant was neutralized to pH 7 with a $NaHCO_3$ (Carlo Erba) solution and I_2 the evolved reduced to HI by $Na_2S_2O_3$ (Carlo Erba). This ether bound (AQU4) fraction was dialysed (Spectrapore 1000 Da) and freeze-dried.

Residual organic matter (RESOM)

The remaining unextracted humic matter (RESOM) was isolated from the soil residue by 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ (Carlo Erba) solution. The supernatant was dialysed (Spectrapore 1000 Da) and freeze-dried.

Liquid chromatography ESI-Orbitrap mass spectrometry

Each Humeome fraction (ORG1, ORG2, ORG3, AQU2, AQU3, AQU4 and RESOM) was dissolved in a 1 mL water and acetonitrile 1:1 solution for a 0.4 g L^{-1} final concentration and added with either 10 µL of LC–MS Grade 25% NH_3 (Romil) solution for the ORG and RESOM fractions, or 10 µL of LC–MS Grade 37% HCl (Romil) solution for the AQU fractions. Samples were injected (50 µL) in a Rheodyne loop in a HPSEC system connected to the LC/MS system. HPSEC comprised a Phenomenex Bio-Sep SEC-S 2000 column (300 × 7.8 mm) and precolumn (30 × 7.8 mm), both thermostatted at 30 °C. A Dionex P 580 pump ensured a 0.3 mL min^{-1} elution of a 55/45 A/B solution (A: 5 mM CH_3COONH_4 in Milli-Q water and 5% CH_3CN, pH 7; B: 100% CH_3CN). Mass spectra were obtained with a LTQ Orbitrap (Thermo Electron, Waltham, MA) and positive ESI, 100–1000 m/z mass range, and 1.0 s scan time. N_2 was the sheath gas (45 AU) and He was the collision gas (7.99 AU). Spray voltage was set at 4.00 kV, spray current at 2.05 µA, capillary temperature at 260 °C, and capillary voltage at 14.93 V.

Data analysis

Masses were translated into molecular formulae using the Excalibur bundled software (Thermo) and setting the following constraints: $P < 1$; $1 > C > 60$; $1 > H > 120$; $0 > O > 60$; $0 > N > 20$ and 5 ppm error. In most cases only one molecular formula fulfilled all requirements. In case of multiple assignments, priority was given to the formulae which could be included in an already existing series of increasing homologues with matching double bond equivalent (DBE) rates. By these criteria, no assignment was ambiguous. The most probable chemical structure for each empirical formula was found by the ChemSpider (http://www.chemspider.com), and the PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases, allowing the assignment to class-specific groups. Each mass peak was integrated for both replicates and means were obtained for all areas under curve (AUC). Semiquantitative assessment (%) was carried out for each peak dividing its AUC by the sum of all AUCs within the specific Humeome fraction. Relative standard deviations were for all data within 10%. Results are reported in Additional file 1: Tables S1–S14.

Results and discussion

The two soils of this study were diverse in texture, pH, and organic carbon content (Table 1). In particular, the silt loam Soil A had a neutral pH while the sandy loam hydromorphic Soil B was slightly acidic. The former showed a larger percentage of organic carbon and higher C/N ratio than the latter.

Compounds in the Humeome and their structural characterization

HPSEC-ESI(+)-Orbitrap-MS analysis enabled the characterization of 175 distinct empirical formulae in Soil A and 139 in Soil B. They were arranged in following three categories: (i) presence (or absence) of N atoms in the empirical formula, henceforth abbreviated in N and no-N; (ii) high or low degree of unsaturation (high or low double bonding equivalent = DBE), abbreviated as HDBE and LDBE; (iii) high or low degree of oxygenation per C atom, abbreviated in HOx and LOx, respectively. Presence of N in a formula was objectively determined, while for the other two criteria the discriminating factors were chosen arbitrarily as it follows: high unsaturation when DBE ≥ 6 and low unsaturation when DBE ≤ 5; high oxygenation when C/O ratio in formulae was ≥ 0.5 and low oxygenation when ≤ 0.5. In case of DBE greater than 10, the abbreviation UHDBE for ultra high unsaturation was used, and formulae showing absence of O atoms were designated by the NOx (no oxygen) abbreviation.

Finally, the empirical formulae were classified into 16 specific chemical groups after identifying their molecular structure based on chemical software programs and databases: aliphatic amides (Aliphatic AD), aromatic amides (Aromatic AD), glycosidic amides (Glycosidic AD), amines (AM), heterocyclic nitrogen compounds (HN), dicarboxylic acids (DA), hydroxylic acids (HA), aliphatic esters (ES), aliphatic ethers (ET), oligosaccharides (saccharide ET), heterocyclic oxygen compounds (HO), phenolic acids (PA), phenolic esters (PE), phenols (PH), steroids (SE), and sterols (ST).

Nitrogenated formulae with low unsaturation and oxygenation degree (N, LDBE, LOx)

All Humeomic fractions of both soils revealed a number of structures characterized by 12 or more C atoms, few unsaturations and typically 4 or occasionally more O atoms. These molecules included mostly a single N atom, even though up to four were found in some formulae. Since these compounds were nearly completely saturated, and given that these humic molecules had likely plant or bacterial origin, it may be inferred that membrane lipids or biopolyesters, such as cutin and suberin, were the most probable precursor for this class of compounds [49]. The main constituents of these biomolecules are either long-chain mono- or di-oic acids, with the chain bearing one or more unsaturations and/or one or more O substituents in the ω or mid-chain position. This suggests that the molecules designated as N, LDBE, LOx revealed (Additional file 1: Tables S1–S14) may derive from the break-down of lipids, cutin and suberin after cell death, and have included nitrogen in the formula at a later stage. ChemSpider and PubChem databases showed that the

main compounds fitting this category were aliphatic AD, and to a lesser extent, some AM and HN.

Nitrogenated formulae with low unsaturation and high oxygenation degree (N, LDBE, HOx)

Molecular structures characterized by the $C_{10–29}$ chain were found in Soil A, and to a much lesser extent in number and amount, in Soil B. These nitrogenated compounds showed few double bonds, but contained several O atoms and were designated as N, LDBE, HOx. The origin of these molecules may be from either a more oxygenated aliphatic cutin and suberin material or a product of lignin breakdown, (Additional file 1: Tables S1–S14). It is inferred that a slow oxidation process may have occurred over time in these compounds and enriched the structure with hydroxyl groups. In fact, the evident anoxic conditions of Soil B have yielded smaller amounts of these structures than the oxic Soil A (Table 3). The application of chemical databases revealed that the main compounds fitting this category were mainly glycosidic AD, and to a much lesser extent, AM.

Nitrogenated formulae with high- or ultra-high-unsaturation and low- or no-oxygenation degree (N, HDBE/UHDBE, LOx/NOx)

Although the low degree of oxygenation of such unsaturated and poorly oxygenated compounds should have suggested a low hydrosolubility of compounds in this category, they remained in the water phase during the extraction due to positively charged nitrogen. Moreover, the much greater number of nitrogen atoms in the UHDBE formulae strongly implied also the presence of heterocyclic N. In case of HDBE formulae, their marked hydrosolubility in the presence of fewer N atoms may be explained with the presence of one or more amino groups instead of heterocycles. While the different substitution pattern may indicate that HDBE and UHDBE formulae may be chemically different, also the chemical databases of both these categories showed a different complexation degree of HN and aromatic AD (Additional file 1: Tables S1, S4–S7 and S11–S14).

Nitrogenated formulae with high unsaturation and oxygenation degree (N, HDBE, HOx)

Hydrosolubility of this group of molecular structures is a consequence of the presence of several oxygen and nitrogen atoms in these formulae. It is likely that these compounds derived from a domain of saccharides bearing different methylation degree, to which they are bound with glycosidic bonds. The chemical databases suggested an abundance of HN, aromatic AD and glycosidic AD compounds (Additional file 1: Tables S4–S7 and S11–S13).

Table 3 Weight yields (%) per type of compound found in the Humeomic fractions

Soil	Class of compound	Fraction						
		ORG1	ORG2	ORG3	AQU2	AQU3	AQU4	RESOM
Soil A	N, LDBE, LOx	49.10	57.54	84.74	0.73	29.72	6.18	2.34
	N, LDBE, HOx	12.70	4.94	3.46	0.02	5.51	–	–
	N, HDBE, LOx	–	0.50	–	55.25	9.53	7.51	8.48
	N, HDBE, HOx	–	–	–	34.68	4.47	0.44	0.26
	N, UHDBE, LOx/NOx	–	–	–	1.47	5.92	13.89	19.43
	Non-N, LDBE, LOx	–	0.69	–	–	4.67	–	0.13
	Non-N, LDBE, HOx	–	2.91	0.63	6.73	20.17	2.01	–
	Non-N, HDBE, LOx	9.16	6.94	0.87	1.12	7.25	10.94	–
	Non-N, HDBE, HOx	29.10	26.48	10.30	–	12.76	59.03	69.36
Soil B	N, LDBE, LOx	75.09	23.16	58.77	–	2.78	62.59	0.56
	N, LDBE, HOx	–	0.83	–	–	1.40	1.78	0.37
	N, HDBE, LOx	–	–	–	69.93	–	–	–
	N, HDBE, HOx	–	–	–	8.47	5.16	7.08	–
	N, UHDBE, LOx/NOx	–	–	–	11.37	7.85	3.76	78.27
	Non-N, LDBE, LOx	2.71	30.21	0.33	1.43	–	10.1	–
	Non-N, LDBE, HOx	–	–	–	0.12	9.87	1.07	–
	Non-N, HDBE, LOx	–	–	–	6.34	8.52	1.07	–
	Non-N, HDBE, HOx	22.20	45.80	40.90	2.34	64.42	12.55	20.80

N nitrogenated, *LDBE/HDBE/UHDBE* low/high/ultra high double bond equivalent, *LOx/HOx/NOx* low/high/no oxygenation

Non-nitrogenated formulae with low unsaturation and oxygenation degree (non-N, LDBE, LOx)

Based on the empirical formulae of this category, the most plausible attribution involves a nearly saturated hydrophobic chain such as those present in compounds commonly found in the MS spectra of humic acids and humin [11, 50]. The great abundance of these oxygenated formulae in Soil B would appear odd, given the anoxic character of this soil. However, it is more likely that this category of compounds was also originally present in Soil A, but underwent oxidation over time, as implied by the prominent abundance of highly oxygenated formulae (non-N, LDBE, HOx) found therein (Table 3). Additionally, the low unsaturation of these formulae is consistent with the less oxidizing environment of the marsh where Soil B was sampled. The application of ChemSpider and PubChem databases suggested the structures of aromatic compounds such as PH, PE and ST, but also of aliphatic compounds such as ES, DA and HA (Additional file 1: Tables S2, S5, S7–S11 and S13).

Non-nitrogenated formulae with low unsaturation and high oxygenation degree (non-N, LDBE, HOx)

As already mentioned, this category of formulae was more numerous and abundant in Soil A than in Soil B, owing to the greater availability of oxygen in the former, which translated into smaller unsaturation and oxygenation (Table 3). The chemical databases for this category indicated structures for aromatic compounds such as PH, PE, and HO. However, a number of structures were also present as ES and ET, and in large amount, as saccharide ET (Additional file 1: Tables S2–S6 and S11–S13).

Non-nitrogenated formulae with high unsaturation and low oxygenation degree (non-N, HDBE, LOx)

These formulae with large degree of unsaturation were found in both soils, although with different distributions. The structures of these molecules entailed a degree of unsaturations that plausibly implied an aromatic nature. Despite high unsaturation and low oxygenation degree, ChemSpider and PubChem databases again assigned the structures to aromatic compounds such as HO, SE, ST, PH, PA and PE (Additional file 1: Tables S1-S3, S5–S6 and S11–S14).

Non-nitrogenated formulae with high unsaturation and high oxygenation degree (non-N, HDBE, HOx)

Compounds characterized by a high degree of unsaturation and oxygenation without N atoms in their formula were revealed by the high-resolution Orbitrap-MS as the most ubiquitous category of compounds. As in previous categories, the chemical databases suggested aromatic compounds, such as PH, PE and HO, also in this category. However, the main constituents were saccharide ET, which they were linked to aromatic moieties, as HO or phenols, in the hydrosoluble fractions (Additional file 1: Tables S1–S3 and S5–S14), thus forming sugar ethers combined to flavonoids or flavanols.

Van Krevelen plots of soil Humeomes

The graphical representation of the MS data for both soils is shown in Van Krevelen diagrams which were built by plotting O/C versus H/C ratios of all detected analytes in any fraction (Fig. 1). Although different components were found in the two soils, they still contained 66 common molecules. It is, thus, noteworthy that despite their geographical distance (about 200 km) and diverse properties (Table 1), similar biochemical and physical–chemical mechanisms are active in stabilizing specific molecules in the two soils.

In the case of the unbound fraction (ORG1), both soils showed that aliphatic AD and saccharide ET were the main chemical groups. However, Soil A comprised 12.7% of glycosylated AD compounds (N, LDBE, HOx formulae in Additional file 1: Table S1), which were totally absent in Soil B (Fig. 2).

The weakly ester-bound organosoluble fraction (ORG2) was similar to ORG1 and contained mainly aliphatic AD and saccharide ET. Nevertheless, the relative percentage of these two groups was inverted in the two soils: Soil A had a larger amount of aliphatic AD, while Soil B was richer in saccharide ET (Fig. 3). Yet, the oxygenated molecules of Soil A were more oxidized, whereas the reduced anoxic molecules of Soil B had a more lipidic character.

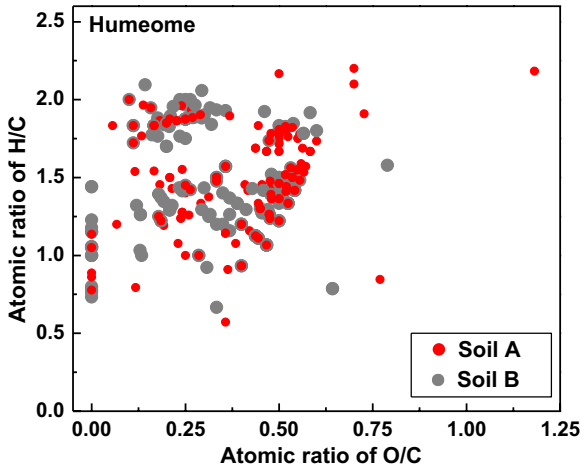

Fig. 1 Van Krevelen plot of total Humeome comprising all molecular structures identified in Soil A (red) and Soil B (grey)

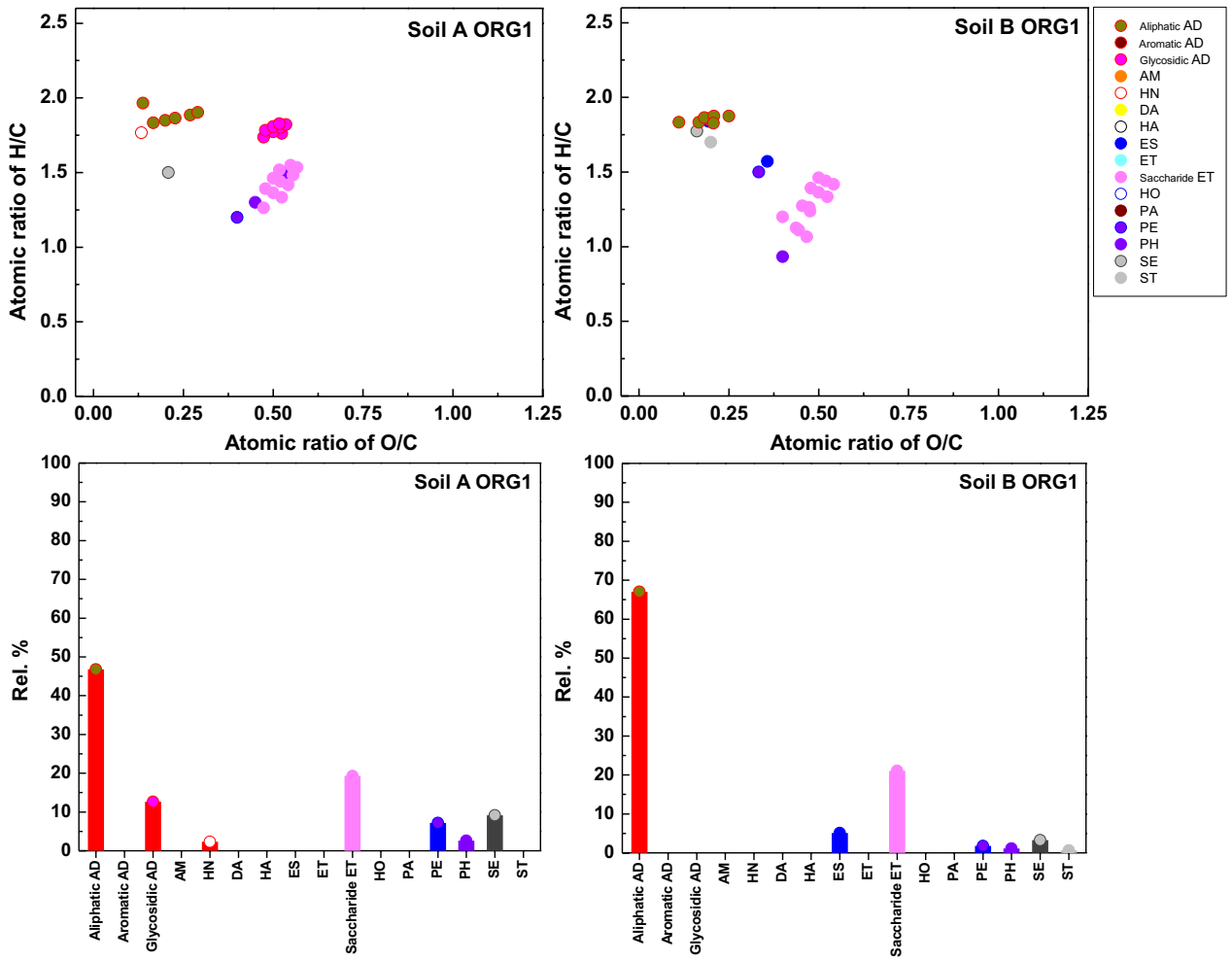

Fig. 2 Van Krevelen plots of molecular structures identified in fraction ORG1 for both soils and their relative percentage distribution in different classes

Aliphatic AD and saccharide ET were again the main compounds found for the strongly ester-bound organosoluble fraction (ORG3). However, the abundance of AM was much larger in Soil A than in Soil B (Fig. 4). It is worth to mention that the silt loam soil (Soil A) had more organosoluble contents than the sandy loam soil (Soil B). This indicates a greater stability of SOM in Soil A, with a consequent larger resistance to oxidation. Another important outcome was that the percentage of common molecules found in both soils was rising from ORG1 to ORG3 (Table 2). This result indicated that the similarity of the two grassland soils is more related to the recalcitrant part of SOM than to the labile fraction.

Soil B showed a larger components' abundance in the hydrosoluble fractions, possibly due to a greater binding capacity of the larger surface for this silt loam soil than for the sandy loam Soil A. However, the hydrosoluble weakly ester-bound fraction (AQU2) was found similar in quantitative abundance in both soils, with aromatic AD and HN compounds being the main representative groups (Fig. 5). Soil A contained partially more oxidized ethers and more reduced aromatic AD than Soil B, that showed instead a larger amount of reduced HN compounds.

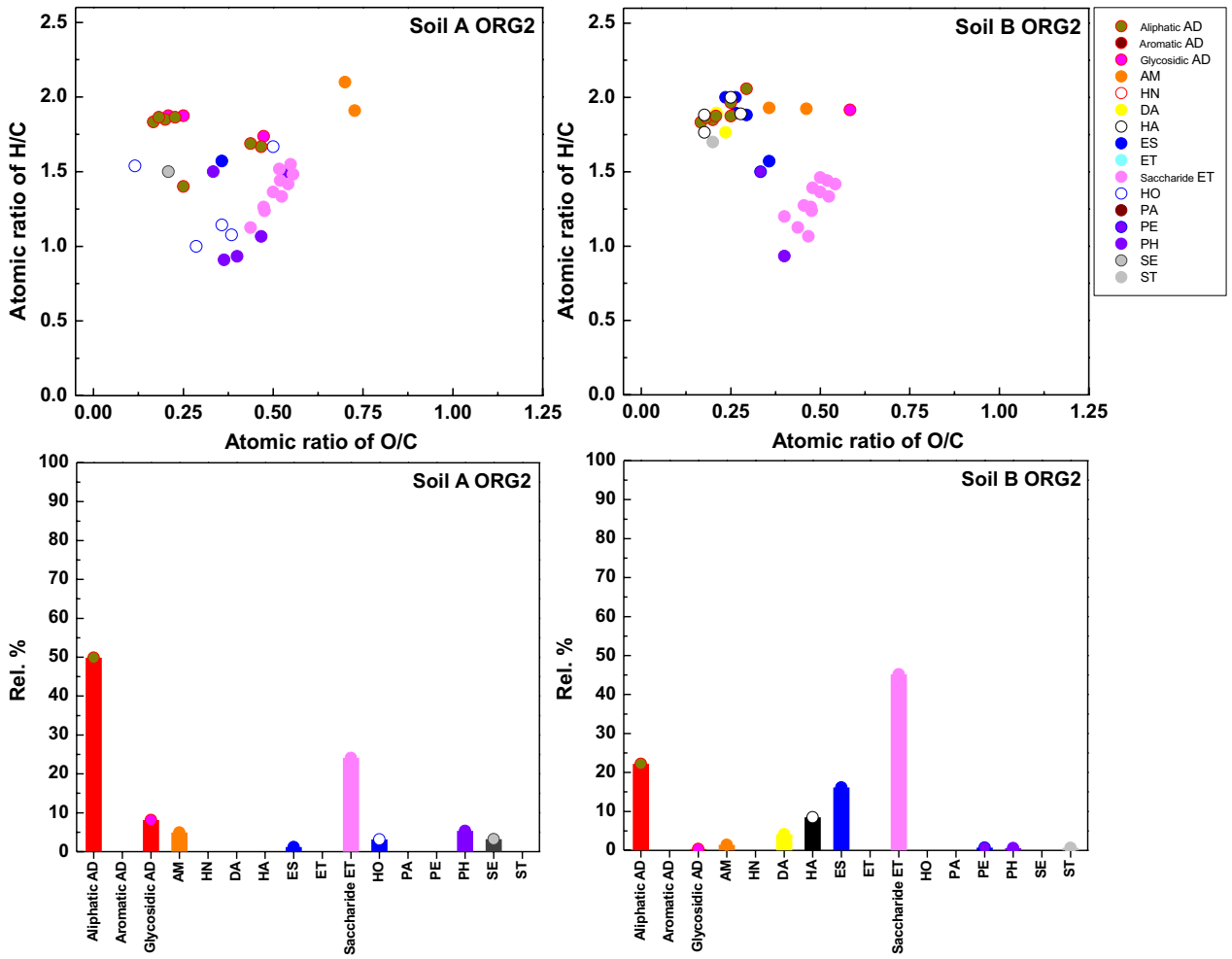

Fig. 3 Van Krevelen plots of molecular structures identified in fraction ORG2 for both soils and their relative percentage distribution in different classes

In the strongly ester-bound fraction (AQU3), the main difference was the greater percentage of Saccharide ET and more reduced HN compounds in Soil B than in Soil A (Fig. 6). Nevertheless, it is important to underline that the mass yield of AQU3 in Soil B was almost 55 times larger than in Soil A (Table 2).

The ether-bound fraction (AQU4) showed a significantly small yield for both soils (Table 2). Nevertheless, Soil B still revealed a larger content of reduced HN compounds than in Soil A and a complete absence of Aromatic AD (Fig. 7).

Finally, the residual organic matter (RESOM) showed similar Van Krevelen plots for both soils (Fig. 8), although the percentage of aromatic AD and saccharide ET as the main groups of compounds in the fraction, was inverted in the two soils. In fact, aromatic AD was more abundant in Soil B, whereas saccharide ET was predominant in Soil A.

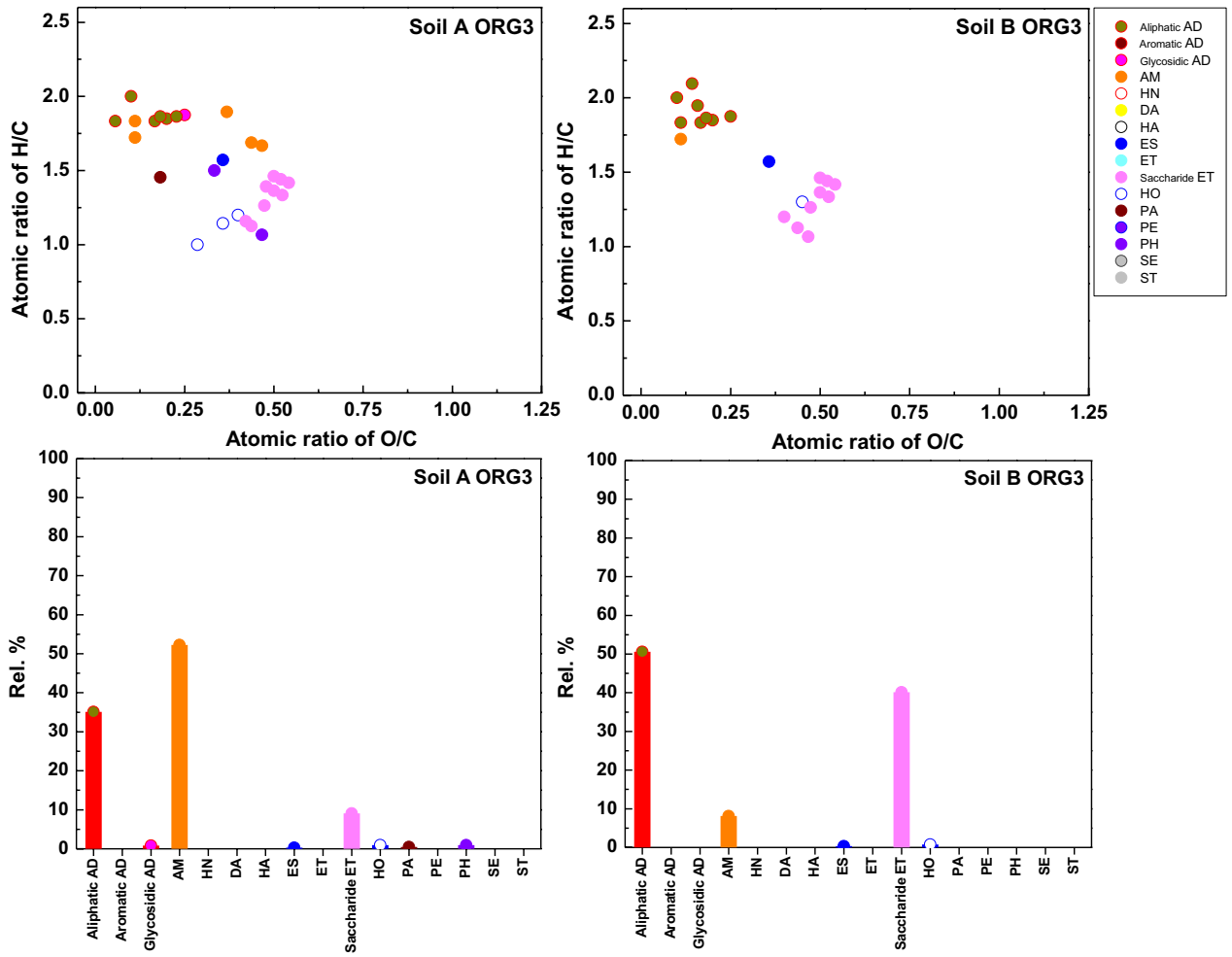

Fig. 4 Van Krevelen plots of molecular structures identified in fraction ORG3 for both soils and their relative percentage distribution in different classes

Conclusions

Two different grassland soils were fractionated here by the Humeomics procedure and the resulting fractions subjected to HPSEC-ESI-Orbitrap-MS characterization. A considerable number of masses was detected and classified according to nitrogenation, oxygenation and unsaturation.

The different properties and environmental conditions of the two grassland soils were reflected in their molecular composition. In particular, the detected masses and their MS relative quantitative assessment indicated a greater nitrogenation of compounds for the hydromorphic sandy loam Soil B and larger oxygenation of components in the oxic silt loam Soil A. The Van Krevelen diagrams built on MS data were proved to be a useful fingerprinting tool to graphically reveal the differences in molecular composition of SOC and SON between the two soils.

The compositional differences among Humeomic fractions were rationalized by taking into account the fact

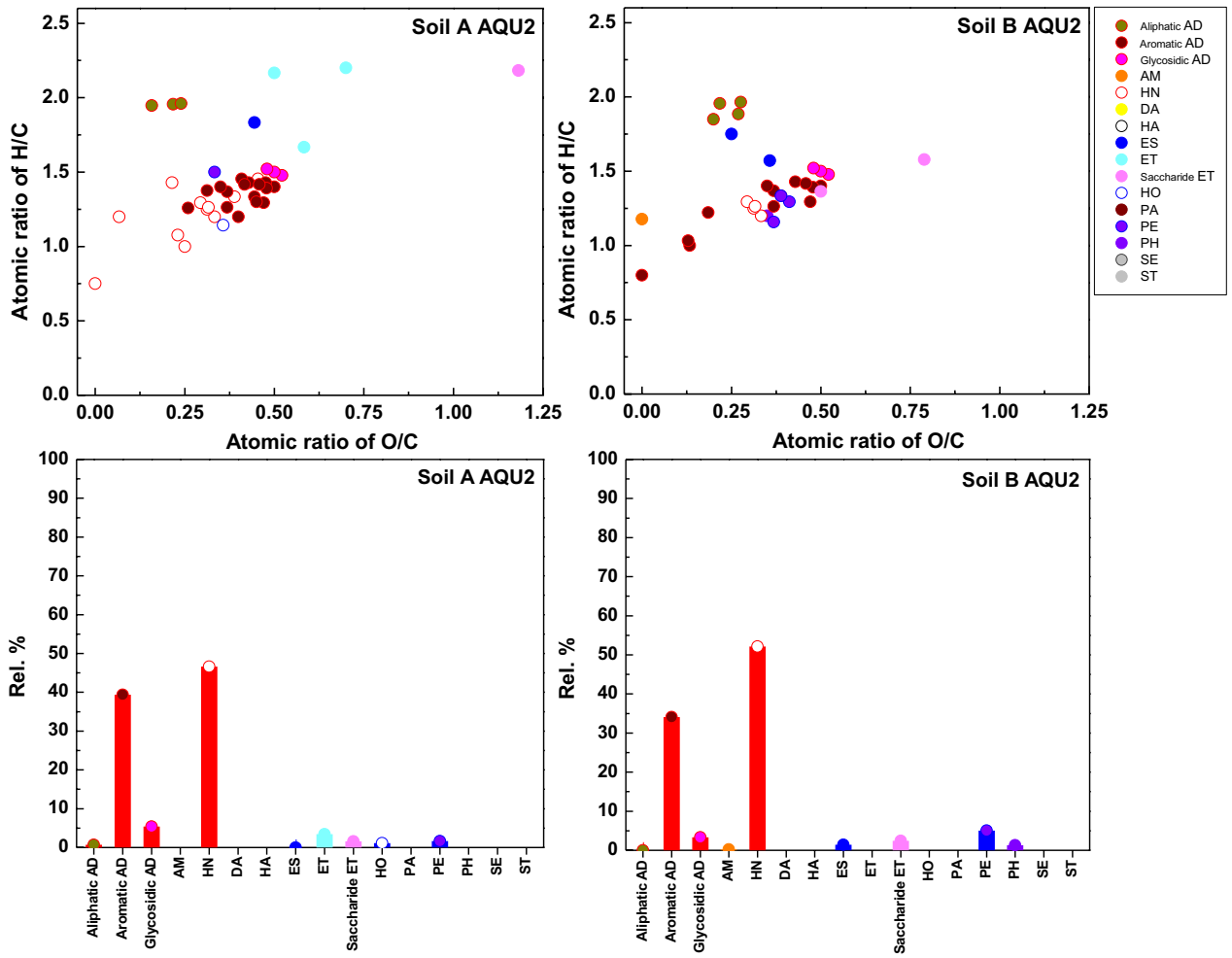

Fig. 5 Van Krevelen plots of molecular structures identified in fraction AQU2 for both soils and their relative percentage distribution in different classes

that carbon–carbon bonds were unaffected, whereas C–O and C–N bonds were subjected to cleavage by hydrolytic reactions of increasing strength (non-covalent > ester > ether/amide). This step-wise approach highlighted the patterns by which some formulae lost the same chemical groups after a specific cleavage, thereby revealing the specific type of chemical bond existing in the humic molecules. This approach, although time consuming, provided unprecedented information on the chemical structure of previously unknown compounds, including the heterocyclic nitrogenated molecules.

It is to be underlined that even though molecular formulae were distributed in statistical categories according to their degree of oxygenation and DBE, most of these categories always resulted into a mixture of different types of compounds. This suggests that interpretations of Van Krevelen results should not be based only on the empirical formulae stemming out from software programs accompanying high-resolution spectrometers, but they should be rather referred to the corresponding molecular structures, as further supported by chemical databases in silico [51]. In fact, careful structural

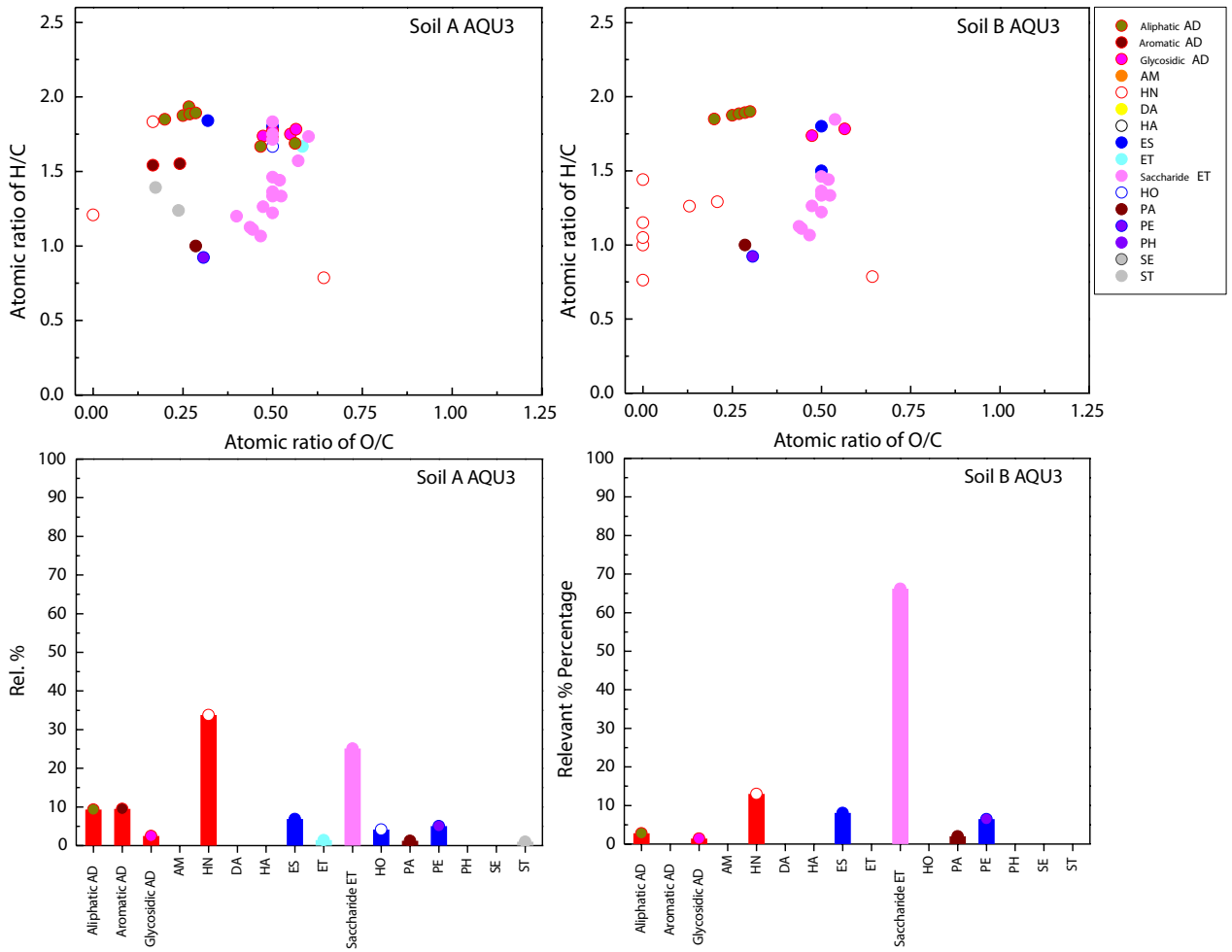

Fig. 6 Van Krevelen plots of molecular structures identified in fraction AQU3 for both soils and their relative percentage distribution in different classes

interpretation by ChemSpider and PubChem databases of empirical formulae obtained from Orbitrap mass spectra revealed that organosoluble fractions of both soils were dominated by aliphatic AD and saccharide ET, while aromatic AD, HN and saccharide ET were predominant in the hydrosoluble fractions. Hence, saccharide ET was found to be the class of compounds common in all types of fractions in both soils, although these components were bound to aromatic moieties in the hydrosoluble fractions. Therefore, these findings indicate that saccharide ET compounds, such as flavanon glycosides, are recalcitrant in grassland soils and represent a stabilized component of SOM.

The mass ratio of the organosoluble to hydrosoluble extracts from the parental soils was 1.9 for the silty Soil A, dropping to 0.9 for the sandy Soil B (Table 2). This difference was due to the high amount of labile ORG1 content present in Soil A, while Soil B was found more abundant in molecules held together by strongly bound esters (AQU3–ORG3) and organominerals (RESOM). However, the relative percentage of compounds being common between the two soils was found to increase

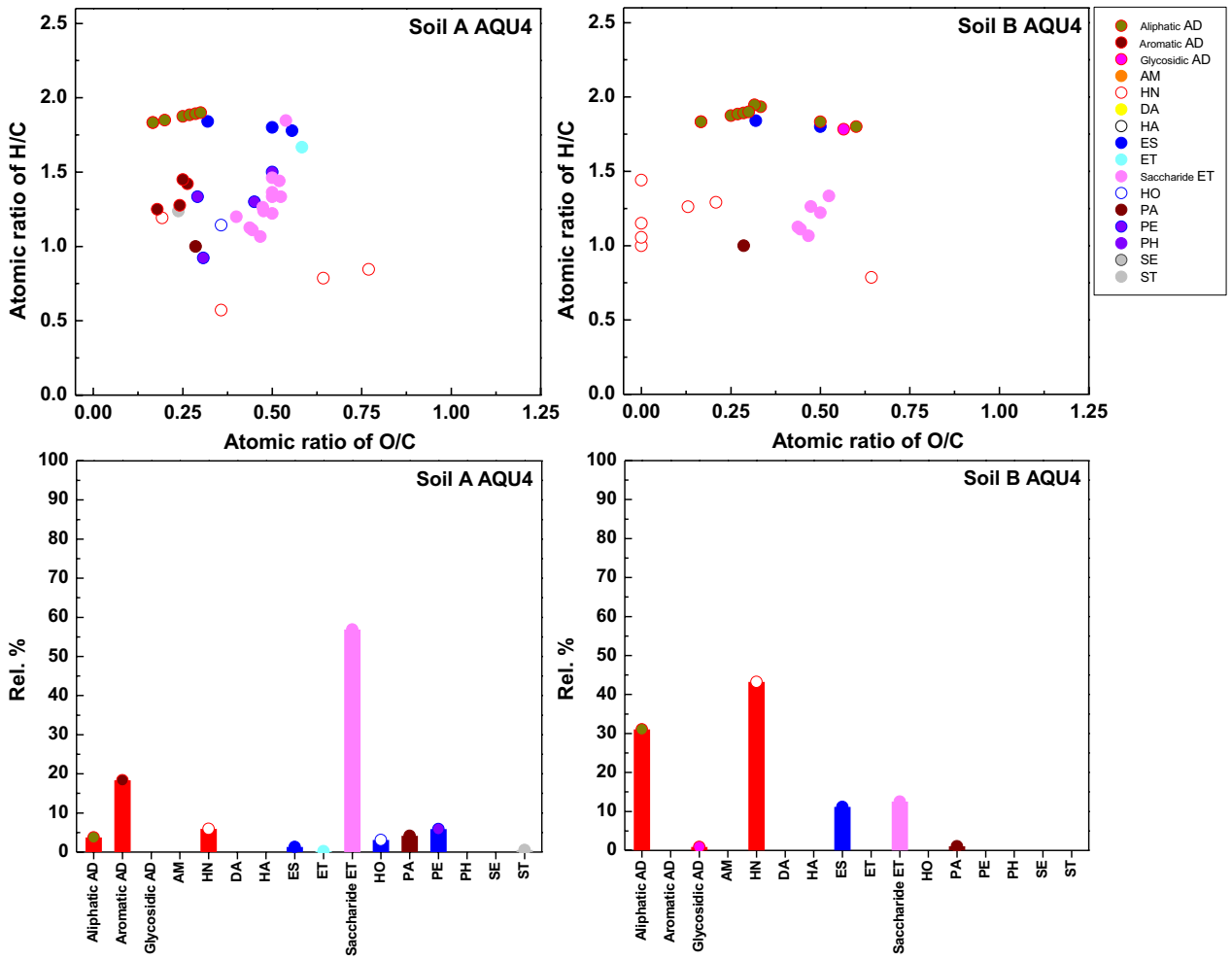

Fig. 7 Van Krevelen plots of molecular structures identified in fraction AQU4 for both soils and their relative percentage distribution in different classes

from ORG1 to ORG3, whereas no such an absolute trend was revealed for the hydrosoluble fractions AQU2-AQU4 and RESOM (Table 2). This result suggests that the stabilization of organic matter in soils is mainly due to its organosoluble fraction, while the hydrosoluble components may be more easily subjected to abiotic and biotic transformations. This conclusion is in line with the supramolecular nature of SOM that implies a stabilization of humus by a progressive accumulation of lipophilic molecules in soil, due to their physical–chemical separation from aqueous media [52]. However, differences in the organosoluble to hydrosoluble mass ratios between the two soils may be related to the varying association of humic molecules with either silt or sand components, thereby forming SOM aggregates of different complexity and stability [53]. Therefore, a further investigation on the humeome of soil aggregates of different dimension and stability may enlarge our knowledge on the mechanisms of SOM sequestration.

The findings of this work indicate that a detailed study of the Humeome of two grassland soils not only

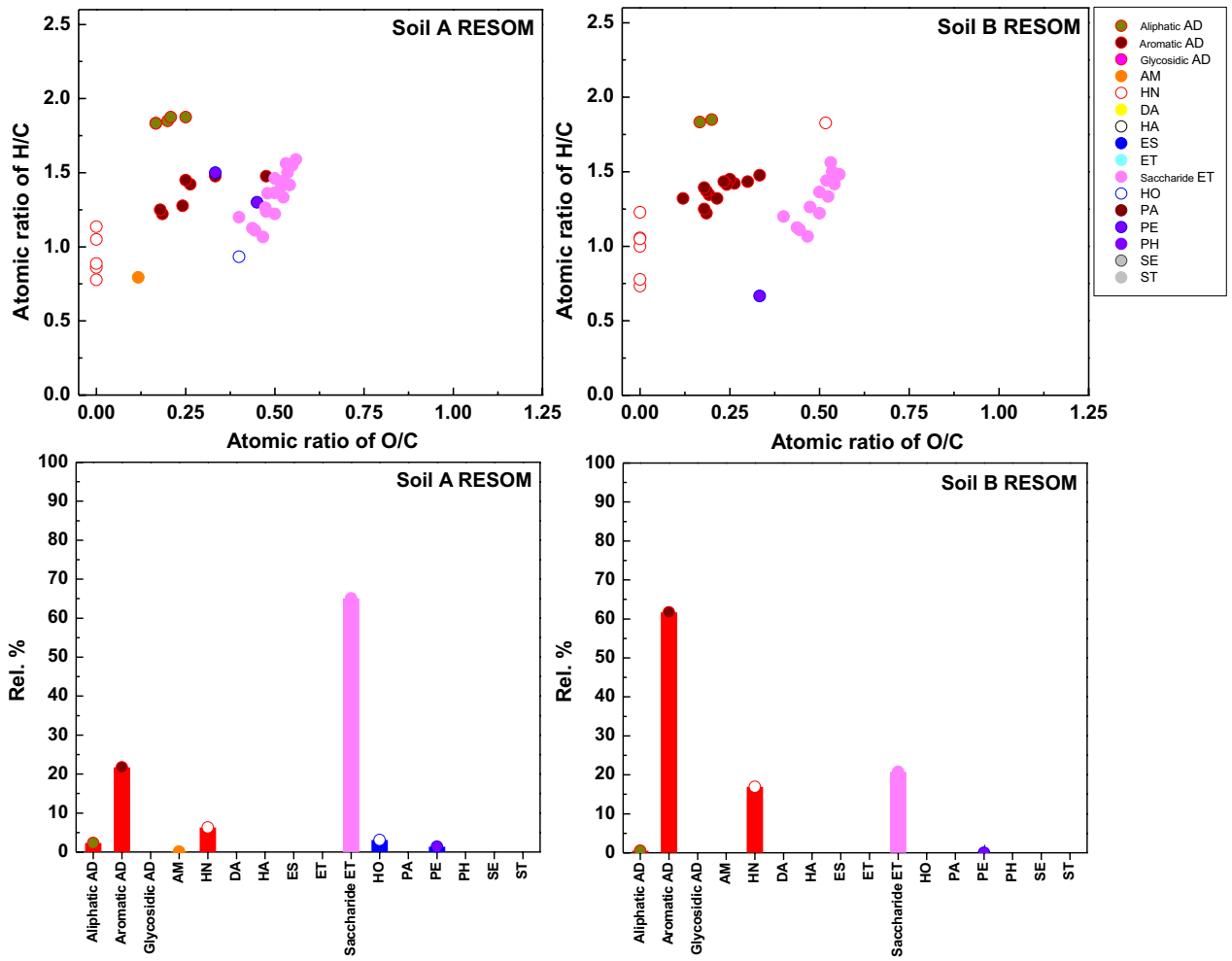

Fig. 8 Van Krevelen plots of molecular structures identified in fraction RESOM for both soils and their relative percentage distribution in different classes

provided unprecedented information on the specific carbon and nitrogen molecular structures in SOM, but also revealed that similar mechanisms are active in the two soils for the long-term stabilization of organic carbon.

Additional file

Additional file 1. Additional tables.

Authors' contributions

All authors have contributed equally to the work. All authors read and approved the final manuscript.

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Acknowledgements

The collaboration of Drs. Antonio Nebbioso and Sebastian Zühlke in obtaining the Orbitrap mass spectra at INFU is gratefully appreciated.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication
All authors give their personal consent for publication.

Ethics approval and consent to participate
Not applicable.

Funding
Not applicable.

Publisher's Note

Received: 14 November 2017 Accepted: 18 June 2018
Published online: 27 July 2018

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