METHODOLOGY

Deriving the Shannon Index from the soil molecular Humeome serves as a descriptor of soil organic matter stability under different cropping systems

Marios Drosos[1*], Michele Orlando[1], Vincenza Cozzolino[2], Antonio Scopa[1*] and Alessandro Piccolo[2]

Abstract

Soil organic matter (SOM) is essential in maintaining soil structure, nutrient content, water retention, and biodiversity. It also represents an important C pool that, if efficiently stabilized, may play a role in the mitigation of global climate changes. However, knowledge is still limited on the dynamics of soil organic carbon (SOC) molecular composition occurring with changes in land use and management practices. We calculated the Shannon diversity index (SH) for the soil molecular characteristics as obtained by the humeomic sequential fractionation applied on two different soil systems including uncultivated control: (1) Soils under a short-term cultivation of maize for 1 and 3 years; (2) Soils under a 20-year long-term cultivation of either continuous maize (MAIZE) or maize-bean rotation (MIX). The SH values were derived for each of the nine molecular classes in which the soil humeomic results were grouped. When single class SH were summed, we found that the resulting total Shannon heterogeneity (SH$_{tot}$) and its value weighted for the relative OC percent, were inversely proportional to a Stability Soil Organic Matter Ratio (SOMR) equation, that, in turn, was directly proportional to the normalized Chemical Protection Ratio (nCPR) equation, based on the quantity of highly hydrophobic (organosoluble) compounds present in soils. The physical–chemical stability of SOM, expressed as SOMR and nCPR, increased with the decrease of the heterogeneity of the total molecular system (SH$_{tot}$) for both short- and long-term soil systems. In fact, in the short-term soil system the molecular humeome was least heterogeneous under uncropped conditions, while SH showed increased heterogeneity with Maize cultivation for 1 and 3 years, in the order. The greatest SH homogeneity was again shown by the uncultivated control soil for the 20-year long-term system, and SH heterogeneity increased passing from the soil under Maize-Broad bean rotation, to that under Maize monocultivation. The SH values of single molecular classes revealed that nitrogenated or oxygenated aromatic compounds determined the stability of soil humus, being the nitrogenated heterocyclic compounds responsible for the residual molecular stability of SOM in both soil systems. This work indicated that the molecular complexity of a soil humeome can be represented by a Shannon descriptor that provides a handy and direct information on the dynamics of humus in soils.

Keywords Soil humeome, Shannon index, Molecular homogeneity, Molecular diversity, Chemical protection ratio, Soil organic matter stability

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Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 2 of 13

Graphical Abstract
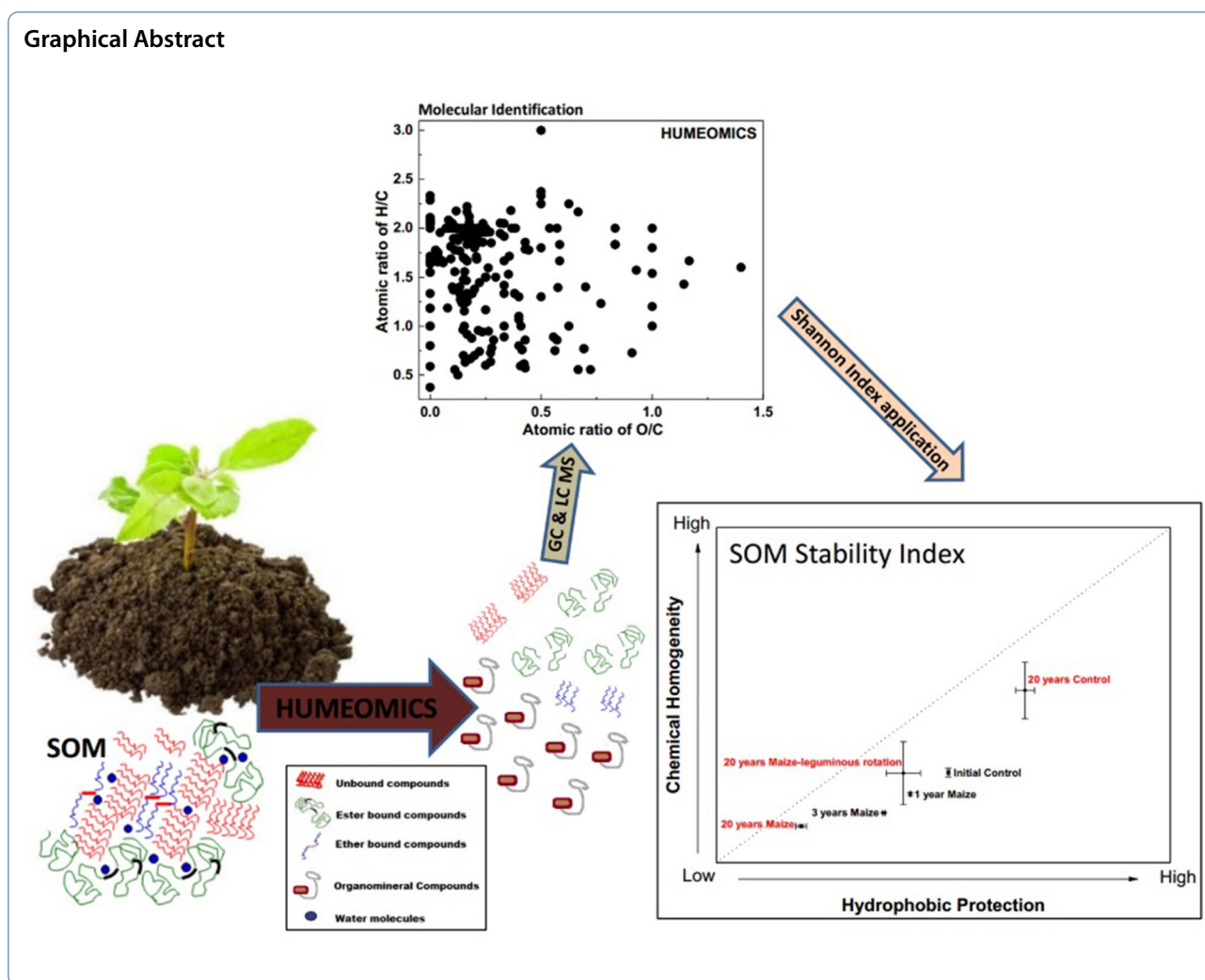
Introduction

Soil is an essential body of the terrestrial ecosystem possessing highly complex physical structures, chemical composition, and biodiversity [1]. The most chemically heterogeneous soil component is soil humus or soil organic matter (SOM) that is the end product of the microbial decomposition of plant litter and animal tissues [2] and is mainly responsible for soil aggregation, water holding capacity, plant nutrient bioavailability, and long-term carbon storage [3]. The capacity to advance in the management of soil humus would represent a giant leap towards a modern sustainable agriculture, since it would allow to control soil erosion and desertification, reduce the use of synthetic fertilizers and pesticides, and limit the emissions of greenhouse gases [4].

However, to achieve these technological goals, it is necessary to improve our understanding of the dynamics of soil humus, by increasing our knowledge on its molecular composition and structural assembly. A consensus has been recently reached in viewing humus as a supramolecular association of a large number of heterogeneous and relatively small molecules which self-assembly due to Van der Walls, $\pi - \pi$, CH–π, and hydrogen bonds [4–7]. Based on this understanding, it was devised a novel chemical fractionation technique of humus, called Humeomic, to obtain more homogeneous humus fractions and facilitate the identification of their molecular composition [8, 9]. The Humeomic fractionation, involves a gradual removal of compounds from the humus matrix by breaking progressively stronger bonds, such as ester and ether bonds, without breaking carbon–carbon bonds [8] and determine their molecular structure by advanced instrumentation such as NMR and mass spectrometry [10–12]. Humeomics was successfully applied on soils to identify, in a tilled agricultural soil, 2.35 times more Organic Carbon (OC)

Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 3 of 13

than that extracted by the common alkaline extraction [13]. Moreover, the OC content of the Humeomic fractions was used to calculate an index related to the physicochemical protection of soil organic matter called Chemical Protection Ratio (CPR) [4]. In fact, the humus hydrophobic domains were found to protect the hydrophilic components from mineralization and microbial degradation [14, 15]. Since the greater the organosoluble/hydrosoluble carbon ratio of the soil humeome, the larger appeared the chemical protection of SOM and its stability [4], it became clear that a further understanding on the stability of soil humus should include a comprehensive knowledge on the homogeneity/heterogeneity of its molecular components.

Among the indices used by the scientific community in the fields of ecology and biology, the Shannon index, or species diversity SH index seems the most applied [16] since it usefully assesses the diversity and dynamics of microbial communities [17, 18]. For example, SH was used to evaluate changes of microbiota during leaf litter decomposition of Quercus Wutaishanica Forest [19–21]. A clear seasonal succession for litter bacterial diversity during decomposition of Quercus Wutaishanica leaves litter was shown by the species diversity index SH, that resulted significantly lower in summer than in other seasons, while the difference between fall, winter and spring was not significant [20].

The Shannon index is useful because it converts into a single value a large amount of data from different samples, which would otherwise be very difficult to compare [22]. Due to the large heterogeneity of components in the supramolecular structure of humus [23], the SH index may allow to reduce the complex variation of different classes of humic molecules and reach a clearer picture of their dynamics in soil. The Shannon index has already been used by Jiménez-González et al., to estimate the pyrolytic heterogeneity of molecular classes present in soil [24]. This study showed significant correlations between the progressive molecular diversity of methoxyphenolic associations and the concentration of organic carbon stored in the corresponding soils. Another work focused on the n-alkanes released by pyrolysis-GC–MS from 35 widely different Mediterranean soils [25]. The SH index was calculated to evaluate the origin and transformations of the homologous series of alkanes (C_9–C_{31}) and a series of multivariate data treatments enabled to show a significant relationship between alkane diversity and SOC concentration. By this method, it was found that the increase in alkane diversity was related to the increase of SOC content and to the resistance of SOM to biodegradation [26]. The SH index has been also applied to the molecular components, as determined by FTICR-MS, of humic and fulvic acids extracted from soils managed for 19 years by either traditional tillage or reduced tillage [27]. Results showed enhanced diversity and chemical richness in the humic extracts composition under reduced tillage.

The aim of this study was to apply the Shannon index to the molecular characteristics of the entire humeome and evaluate the molecular stability of organic matter in soils under different land use and management. We hypothesized that a more stable soil humus system may consist of more homogeneous components in the soil humeome.

Methods

Soil

A Typic Ustifluvent (63% sand, 30% silt and 7% clay classified as a sandy loam soil with pH 8) under continuous Maize (*Zea mais* L.) in the experimental station of the University of Torino [13] was used for the short-term cultivation experiment with three samplings at a depth of 0–30 cm deep: (i) uncultivated control soil (Initial Control); (ii) soil after one year of cultivation (MAIZE 1); (iii) soil after three years of cultivation (MAIZE 3). Details on land management and sampling times are reported in [28].
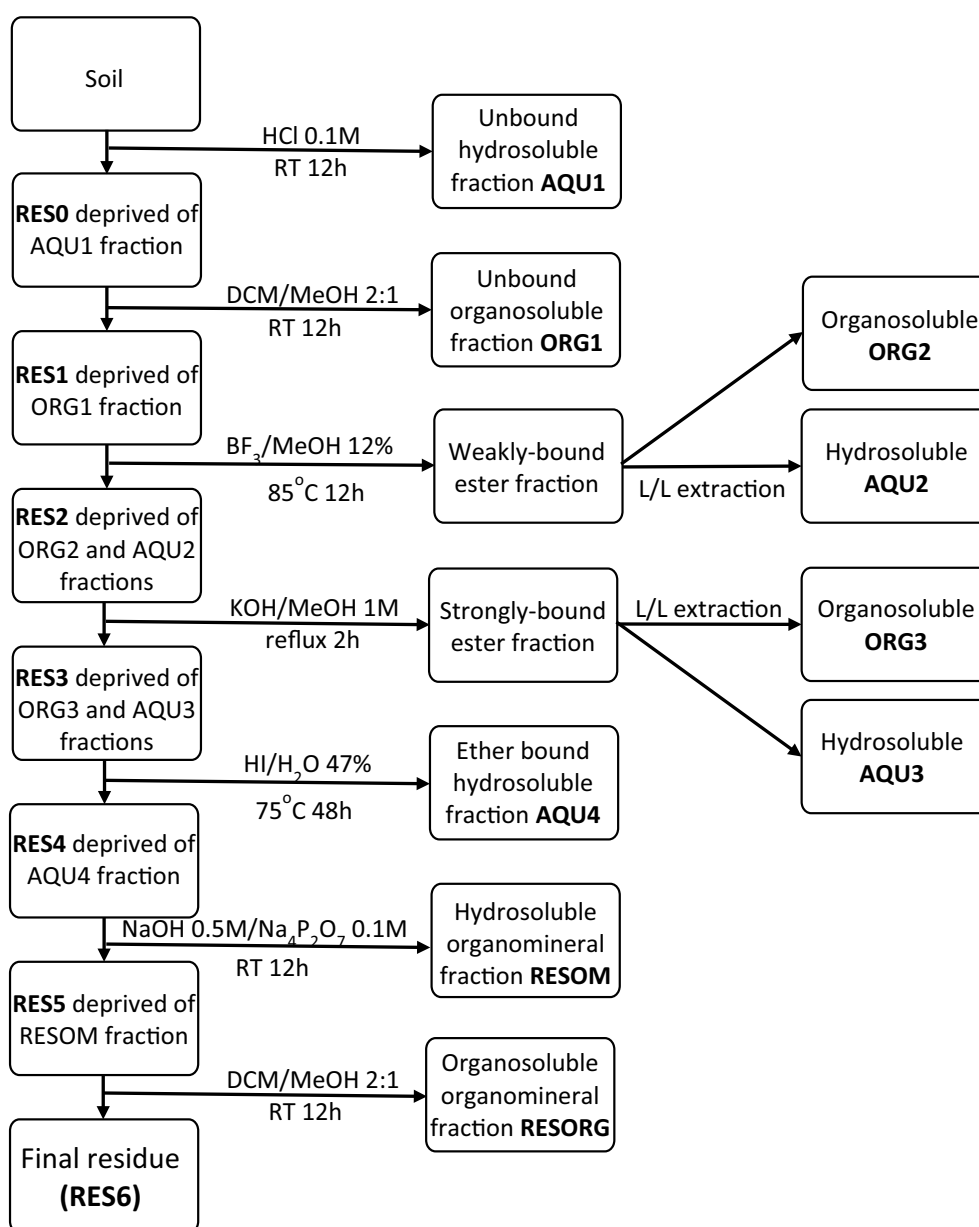
The long-term cultivation experiment was conducted at the experimental farm of the University of Napoli Federico II at Castel Volturno, on a Vertic Xerofluvent (18.3% sand, 31.3% silt, and 50.4% clay classified as a silty-clay loam soil with pH 8.3) with sampling at a depth of 20 cm deep. This soil was permanently managed under three different cropping systems for 20 years: (i) untilled control soil without cultivation (Control); (ii) Maize monoculture (MAIZE); (iii) Maize-Broad bean (*Vicia faba* L.) rotation (MIX). Details are reported in [29].

Humeomics sequential fractionation

The Humeomics fractionation was applied to the soils of the two different management systems, as previously described [13, 28, 29], to obtain a series of organosoluble and hydrosoluble separated fractions: unbound fraction (ORG1), weakly ester-bound fractions (ORG2 and AQU2), strongly ester-bound fractions (ORG3 and AQU3), strongly ether-bound fraction (AQU4), and extraction from the final residual soil to obtain further hydrosoluble (RESOM) and organosoluble (RESORG) fractions (Scheme 1).

Fractions characterization

The organosoluble fractions (ORG 1–3 & RESORG) were analyzed by GC–MS, while the molecular composition of hydrosoluble fractions (AQU 2–4 & RESOM) was characterized by High Resolution ESI-Orbitrap-MS [13, 28, 29]. The identified molecules were grouped in nine main groups: organic nitrogen compounds such

Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 4 of 13

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┌──────────────┐
│     Soil     │
└──────────────┘
        │         HCl 0.1M          ┌──────────────────┐
        │──────────────────────────▶│     Unbound       │
        │         RT 12h            │ hydrosoluble      │
        ▼                           │ fraction AQU1     │
┌──────────────┐                    └──────────────────┘
│ RES0 deprived│
│  of AQU1     │
│  fraction    │
└──────────────┘
        │      DCM/MeOH 2:1         ┌──────────────────┐
        │──────────────────────────▶│     Unbound       │
        │         RT 12h            │ organosoluble     │     ┌──────────────────┐
        ▼                           │ fraction ORG1     │     │  Organosoluble   │
┌──────────────┐                    └──────────────────┘   ┌▶│      ORG2        │
│ RES1 deprived│                                            │ └──────────────────┘
│  of ORG1     │                                            │
│  fraction    │                                            │
└──────────────┘                    ┌──────────────────┐   │
        │      BF3/MeOH 12%         │  Weakly-bound    │───┘ ┌──────────────────┐
        │──────────────────────────▶│  ester fraction  │     │  Hydrosoluble    │
        │        85°C 12h           │                  │────▶│      AQU2        │
        ▼                           └──────────────────┘ L/L └──────────────────┘
┌──────────────┐                                      extraction
│ RES2 deprived│
│ of ORG2 and  │
│ AQU2         │
│ fractions    │
└──────────────┘
        │      KOH/MeOH 1M          ┌──────────────────┐  L/L ┌──────────────────┐
        │──────────────────────────▶│  Strongly-bound  │extraction Organosoluble │
        │        reflux 2h          │  ester fraction  │────▶│      ORG3        │
        ▼                           └──────────────────┘     └──────────────────┘
┌──────────────┐                            │
│ RES3 deprived│                            │                ┌──────────────────┐
│ of ORG3 and  │                            └───────────────▶│  Hydrosoluble    │
│ AQU3         │                                             │      AQU3        │
│ fractions    │                                             └──────────────────┘
└──────────────┘
        │      HI/H2O 47%           ┌──────────────────┐
        │──────────────────────────▶│   Ether bound    │
        │        75°C 48h           │  hydrosoluble    │
        ▼                           │ fraction AQU4    │
┌──────────────┐                    └──────────────────┘
│ RES4 deprived│
│  of AQU4     │
│  fraction    │
└──────────────┘
        │  NaOH 0.5M/Na4P2O7 0.1M   ┌──────────────────┐
        │──────────────────────────▶│   Hydrosoluble   │
        │         RT 12h            │  organomineral   │
        ▼                           │ fraction RESOM   │
┌──────────────┐                    └──────────────────┘
│ RES5 deprived│
│  of RESOM    │
│  fraction    │
└──────────────┘
        │      DCM/MeOH 2:1         ┌──────────────────┐
        │──────────────────────────▶│  Organosoluble   │
        │         RT 12h            │  organomineral   │
        ▼                           │ fraction RESORG  │
┌──────────────┐                    └──────────────────┘
│Final residue │
│   (RES6)     │
└──────────────┘
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Scheme 1 Humeomics sequential fractionation protocol applied directly in Soil

as (i) aminoacids, amines and imines (AA/AM/IM), (ii) amides (AD), and, (iii) heterocyclic nitrogen compounds (HN); aromatic compounds comprising (iv) phenolic acids, phenolic esters/ethers and phenols (PA/PE/PH), and, (v) benzoic acids, benzoic esters/ethers, heterocyclic oxygen compounds and polyaromatic hydrocarbons (BA/BE/HO/PAH); (vi) resin acids, steroids and sterols (RA/SE/ST); aliphatic acids such as (vii) fatty, dicarboxylic and hydroxyl acids (FA/DA/HA); sugar derivatives including (viii) sugar acids, sugar esters/ethers, and sugars (SA/SET/SU); and other aliphatic compounds such as (ix) esters, ethers, ketones, alcohols, aldehydes and alkanes/alkenes/alkynes (ES/ET/KE/AC/AH/AL). The Shannon index was applied to the concentrations of identified molecules grouped into these molecular classes to obtain a single number that would represent an indication of molecular diversity among the groups' compounds of each considered soil system and practice.
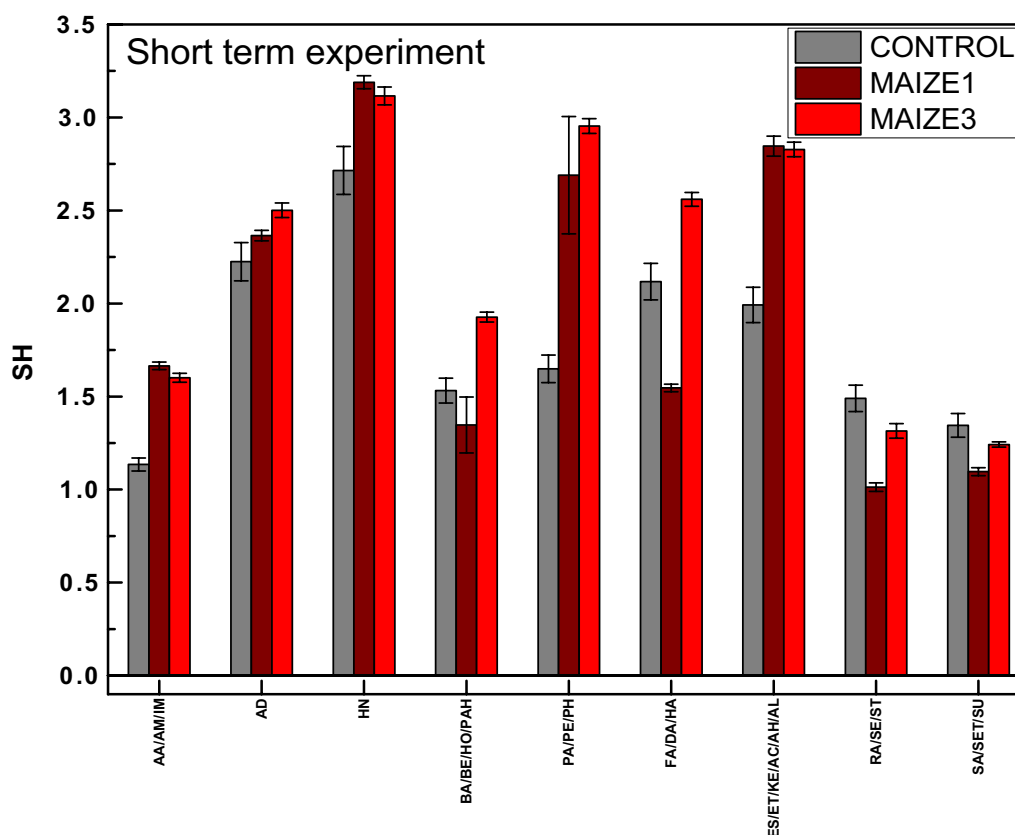
Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 5 of 13

Fig. 1 Shannon (SH) Index derived from 9 molecular classes identified by soil humeomic fractionation applied to soils of the short-term experiment: uncropped (Control), under 1 year of Maize cultivation (Maize 1), and under 3 years of Maize cultivation (Maize 3). AA = amino acids; AM = amines; IM = imines; AD = amides; BA = benzoic acids; BE = benzoic esters; HO = heterocyclic oxygen compounds; PAH = polycyclic aromatic hydrocarbons; PA = phenolic acids; PE = phenolic esters; PH = phenols; FA = fatty acids; DA = dicarboxylic acids; HA = hydroxy acids; ES = aliphatic esters; ET = ethers; KE = ketones; AC = alcohols; AH = aldehydes; AL = alkanes/alkenes/alkynes; RA = resin acids; SE = steroids; ST = sterols; SA = sugar acids; SET = sugar esters; SU = sugars; HN = heterocyclic nitrogen compounds

Shannon Index

The Shannon index is usually used to measure the diversity of species in a microbial community in eco-biological studies [30–32]. This index is indicated with SH and is calculated from the expression:

$$SH = - \sum \left[(p_i) \times \ln (p_i) \right] \quad (1)$$

where p_i is the summation from $i = 1$ to $i = n$ of the relative quantity of i species in a whole community, multiplied by the natural logarithm of the same quantity and changed in sign. This SH index is directly proportional to the number of species and the uniformity of their abundance. The minimum value is 0 when only one species is present within the community, whereas the maximum value is equal to ln (k) where k is the number of species, i.e., there are k species with the same number of individuals.

To derive the Shannon index from the molecular classes of a soil humeome, we substituted biological species with molecular classes, and individuals with concentrations of single molecules belonging to a specific molecular class. We calculated the species diversity as an SH index by the *Past 4.11* free software with biased version. In fact, if under-sampling is suspected, the actual number of species based on Shannon entropy controlled by bias is the most suitable measure of diversity [33]. In practice, SH was derived from the concentrations, expressed as percent of organic carbon, of the molecules found in the humeome of the studied soil systems [13, 28, 29], which were grouped into 9 main classes of compounds. The percent of organic carbon was calculated by dividing each molecular concentration by the total organic carbon extracted from soil through the Humeomic fractionation. The % OC of each molecule, belonging to the different compounds classes, was placed into the *Past 4.11* software to calculate the Shannon index.

Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 6 of 13

Table 1 Shannon index (SH), SH multiplied by the % OC in the specific molecular class, and their respective total values (SH$_{tot}$) for all the molecular classes, derived from the molecular soil humeome for Control, Maize after 1 year (Maize 1) and 3 years cultivation (Maize 3) of the short-term experiment

Molecular classes[a]	CONTROL[b]		MAIZE 1[c]		MAIZE 3[c]	
	SH	SH×%OC	SH	SH×%OC	SH	SH×%OC
AA/AM/IM	1.135±0.035	0.033±0.001	1.665±0.020	0.169±0.002	1.601±0.024	0.132±0.002
AD	2.225±0.103	0.043±0.002	2.365±0.028	0.515±0.006	2.501±0.039	0.576±0.009
BA/BE/HO/PAH	1.532±0.067	0.160±0.007	1.347±0.150	0.009±0.001	1.927±0.027	0.071±0.001
PA/PE/PH	1.649±0.074	0.134±0.006	2.69±0.315	0.188±0.022	2.954±0.040	0.295±0.004
FA/DA/HA	2.118±0.098	0.562±0.026	1.546±0.020	0.394±0.005	2.560±0.037	0.491±0.007
ES/ET/KE/AC/AH/AL	1.992±0.095	0.672±0.032	2.846±0.054	0.106±0.002	2.828±0.039	0.145±0.002
RA/SE/ST	1.490±0.071	0.021±0.001	1.013±0.023	0.044±0.001	1.315±0.039	0.034±0.001
SA/SET/SU	1.345±0.064	0.127±0.006	1.096±0.021	0.104±0.002	1.242±0.014	0.089±0.001
HN	2.715±0.129	0.147±0.007	3.189±0.035	0.553±0.006	3.116±0.048	0.656±0.010
SH$_{tot}$	16.201±0.751	1.899±0.088	17.757±0.401	2.082±0.047	20.044±0.298	2.489±0.037
nCPR	0.511±0.003		0.427±0.001		0.369±0.003	
SOMR	0.269±0.013		0.205±0.005		0.148±0.002	

The SH and the SH x %OC values were used to calculate the nCPR and SOMR ratios, as described in "Methods" section. Standard deviation was derived by analysis in triplicates

[a] AA = amino acids; AM = amines; IM = imines; AD = amides; BA = benzoic acids; BE = benzoic esters; HO = heterocyclic oxygen compounds; PAH = polycyclic aromatic hydrocarbons; PA = phenolic acids; PE = phenolic esters; PH = phenols; FA = fatty acids; DA = dicarboxylic acids; HA = hydroxy acids; ES = aliphatic esters; ET = ethers; KE = ketones; AC = alcohols; AH = aldehydes; AL = alkanes/alkenes/alkynes; RA = resin acids; SE = steroids; ST = sterols; SA = sugar acids; SET = sugar esters; SU = sugars; HN = heterocyclic nitrogen compounds

[b] Based on the Humeomics fractionation presented by Drosos et al. (2017) in [13]

[c] Based on the Humeomics fractionation presented by Drosos and Piccolo (2018) in [28]

From Shannon index to stability organic matter ratio

A direct relationship between an enhanced molecular hydrophobicity and the organic matter protection was previously noted [4]. This relation was shown by the Chemical Protection Ratio (CPR) based on the ratio of the total OC found in all organosoluble fractions divided by the total OC found in all hydrosoluble fractions separated from soil by the Humeomic procedure:

$$CPR = ORG_n / (AQU_n + RESOM) \qquad (2)$$

In this study, we normalized this ratio (nCPR) by dividing the total OC found in all organosoluble fractions by the total OC found in all fractions separated from soil by the Humeomic procedure:

$$\begin{aligned} nCPR = (ORG_n + RESORG) \\ /(ORG_n + RESORG \\ + AQU_n + RESOM) \end{aligned} \qquad (3)$$

The Shannon indices calculated by the Eq. (1) for each of the nine molecular classes obtained for any single soil of the two systems considered here, were combined to obtain the total Shannon index (SH$_{tot}$) that indicates the overall heterogeneity of each soil molecular humeome.

The SH$_{tot}$ and nCPR were then used to introduce a new index named Stability Organic Matter Ratio (SOMR):

$$SOMR = nCPR / (SH_{tot} \times \%OC) \qquad (4)$$

SOMR was, therefore, based on results of Humeomic fractionation of the different soil humeomes and concomitantly indicated both the heterogeneity and hydrophobicity of the molecular systems in soil humus. Equation 4 suggests that the soil stability is directly proportional to the content of hydrophobic molecules (nCPR), and inversely proportional to the sum of SH indices (SH$_{tot}$).

Results and discussion

Soil system under short-term cultivation

The Shannon Index for the differently cropped soils of the short-term cultivation experiment (Fig. 1; Table 1) were derived from the data obtained by the humeomic fractionation previously applied to the soils [13, 28]. From the same data of soil humeomes [13, 28], the nCPR ratio derived here shows that the hydrophobicity of soil humus decreased with the extent of maize cultivation. In fact, the nCPR values diminished progressively passing from 0.511 of the control soil, to 0.427 and 0.369 of the soils under MAIZE1 and MAIZE3, respectively
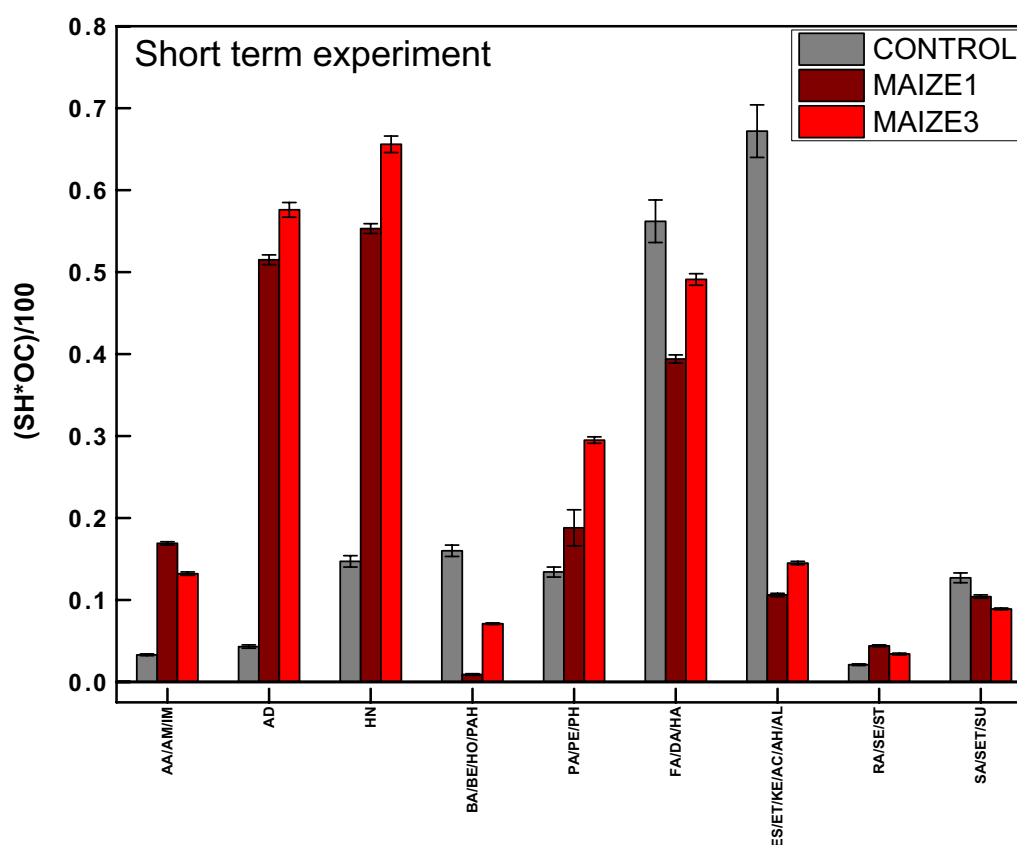
Fig. 2 Shannon (SH) Index weighted for % OC in the nine molecular classes identified by soil humeomic fractionation applied to soils of the short-term experiment: uncropped (Control), under 1 year of Maize cultivation (Maize 1), and under 3 years of Maize cultivation (Maize 3). AA = amino acids; AM = amines; IM = imines; AD = amides; BA = benzoic acids; BE = benzoic esters; HO = heterocyclic oxygen compounds; PAH = polycyclic aromatic hydrocarbons; PA = phenolic acids; PE = phenolic esters; PH = phenols; FA = fatty acids; DA = dicarboxylic acids; HA = hydroxy acids; ES = aliphatic esters; ET = ethers; KE = ketones; AC = alcohols; AH = aldehydes; AL = alkanes/alkenes/alkynes; RA = resin acids; SE = steroids; ST = sterols; SA = sugar acids; SET = sugar esters; SU = sugars; HN = heterocyclic nitrogen compounds

(Table 1). Here, the diversity SH values obtained for the nine molecular classes allowed to calculate the SH_{tot}, that revealed, for the first time, that the heterogeneity of the molecular system in the soil humeome showed an opposite trend to the soil hydrophobicity, since it increased with the number of years of Maize cultivation (Table 1). The least heterogeneous system resulted the uncultivated control soil with a SH_{tot} of 16.201, while 1.899 was the value when the SH_{tot} heterogeneity was weighted on the percent of organic carbon in all molecular classes ($SH_{tot} \times$ % OC). These two values increased to 17.757 and 2.082, respectively, for MAIZE1 and up to 20.044 and 2.489 for MAIZE3 (Table 1).

Hence, a detailed molecular information given by the humeomic fractionation of soils allows to derive two indices of hydrophobicity and heterogeneity of soil humic molecules which are related to the overall molecular stability of SOM. These parameters indicate that soil organic matter becomes less stable, and therefore more prone to degradation/mineralization, when a decrease of its

molecular hydrophobicity is concomitant to an increase of its molecular heterogeneity. A reduced molecular stability of SOM following a decline in the degree of humus hydrophobicity with soil perturbation due to tillage and cropping, had been already reported, albeit using physical–chemical results [34] or several indirect soil biological properties [35]. Moreover, the nCPR and the SH_{tot} indices are combined here to obtain the SOMR index, whose values indicate the overall molecular stability of soil organic matter based on the molecular characteristics of soil humus. The values of SOMR, when applied to the soil system under short-term cultivation, suggest that the molecular stability of SOM continues to decrease with the number of years of Maize cultivation. In fact, SOMR was reduced from 0.269 of the uncultivated control soil to 0.205 and even 0.148 for the MAIZE1 and MAIZE3 cropped soils, respectively (Table 1). In fact, molecular stability of soil humus can be related to its hydrophobic properties which stabilize and accumulate heterogeneous organic molecules in soil because of a mechanism of

Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 8 of 13

Table 2 Shannon index (SH), SH multiplied by the % OC in the specific molecular class, and their respective total values (SH$_{tot}$) for all the molecular classes, derived from the molecular soil humeome for Control, MIX and Maize of the long-term experiment

Molecular classes[a]	CONTROL[b]		MIX[b]		MAIZE[b]	
	SH	SH×%OC	SH	SH×%OC	SH	SH×%OC
AA/AM/IM	0.910±0.129	0.141±0.020	1.167±0.267	0.144±0.033	1.055±0.019	0.221±0.004
AD	1.380±0.197	0.098±0.014	2.040±0.463	0.172±0.039	2.635±0.052	0.202±0.004
BA/BE/HO/PAH	1.449±0.193	0.030±0.004	1.608±0.342	0.080±0.017	1.361±0.034	0.080±0.002
PA/PE/PH	1.783±0.248	0.115±0.016	2.307±0.528	0.131±0.030	2.422±0.030	0.164±0.002
FA/DA/HA	2.098±0.298	0.345±0.049	2.206±0.493	0.273±0.061	2.156±0.036	0.238±0.004
ES/ET/KE/AC/AH/AL	1.044±0.148	0.212±0.030	1.301±0.295	0.194±0.044	1.161±0.020	0.118±0.002
RA/SE/ST	0.404±0.050	0.032±0.004	0.086±0.014	0.006±0.001	0.129±0.022	0.006±0.001
SA/SET/SU	1.136±0.156	0.153±0.021	0.975±0.756	0.098±0.076	1.224±0.028	0.043±0.001
HN	1.861±0.265	0.197±0.028	1.822±0.410	0.444±0.100	2.165±0.037	0.642±0.011
SH$_{tot}$	12.064±1.696	1.323±0.186	13.512±3.512	1.543±0.401	14.308±0.259	1.714±0.031
nCPR	0.680±0.021		0.412±0.038		0.187±0.012	
SOMR	0.514±0.084		0.267±0.094		0.109±0.005	

The SH and the SH x %OC values were used to calculate the nCPR and SOMR ratios, as described in "Methods" section. Standard deviation was derived by analysis in triplicates

[a] AA=amino acids; AM=amines; IM=imines; AD=amides; BA=benzoic acids; BE=benzoic esters; HO=heterocyclic oxygen compounds; PAH=polycyclic aromatic hydrocarbons; PA=phenolic acids; PE=phenolic esters; PH=phenols; FA=fatty acids; DA=dicarboxylic acids; HA=hydroxy acids; ES=aliphatic esters; ET=ethers; KE=ketones; AC=alcohols; AH=aldehydes; AL=alkanes/alkenes/alkynes; RA=resin acids; SE=steroids; ST=sterols; SA=sugar acids; SET=sugar esters; SU=sugars; HN=heterocyclic nitrogen compounds

[b] Based on the Humeomics fractionation presented by Savarese et al. (2021) in [29]

physical–chemical protection [4, 14, 23] that excludes a large part of humic matter from biotic and abiotic oxidative degradation. Once soil humus undergoes oxidative processes that degrade its hydrophobicity (tillage, continuous cultivation, etc.), it also loses its molecular homogeneity, and, concomitantly, its molecular stability [14, 23].

The Shannon diversity index applied to the molecular results of the humeomic analysis of soils is also capable to indicate which single molecular class preferably contributes to changes of soil organic matter stability during soil cropping. In the case of the soil under the short-term experiment, amides (AD); phenolic acids, phenolic esters/ethers and phenols (PA/PE/PH); esters, ethers, ketones, alcohols, aldehydes and alkanes/alkenes/alkynes (ES/ET/KE/AC/AH/AL); and heterocyclic nitrogen compounds (HN), were the compounds classes which mostly determined SOM stability (Fig. 1). Their SH values, and, thus, their heterogeneity increased steadily passing from the uncropped control soil to MAIZE1 and MAIZE3 soils (Fig. 1). The PA/PE/PH molecular class, a product of lignin degradation in soil, and the ES/ET/KE/AC/AH/AL group of compounds provided a Shannon index that significantly increased with years of Maize cultivation (Fig. 1). In fact, while SH for PA/PE/PH molecular class passed from 1.649 of uncultivated soil to 2.69 and 2.954 for MAIZE1 and MAIZE3 soils, respectively, the ES/ET/KE/AC/AH/AL class of compounds provided SH values which increased from 1.992 to 2.846 and 2.828

for the same soil treatments of the short-term experiment (Fig. 1, Table 1). The concomitant heterogeneity enhancement of these molecular classes with reduction of the molecular stability of SOM is in line with previous reports [24, 36].

In the case of amides, the SH heterogeneity weighted by the OC percent, increased from 0.043 for the uncultivated control soil to 0.515 and 0.576 for the respective soils under 1 and 3 years of maize cultivation (Fig. 2; Table 1). A similar increase was observed for the HN compounds, whose weighted heterogeneity by OC percent increased to 0.553 and 0.656 for the MAIZE1 and MAIZE3 soils, respectively, as compared to 0.147 in the case of the uncultivated control soil (Fig. 2; Table 1). This trend of both AD and HN classes indicates that in a soil with increasing maize cultivation, the loss of SOM molecular stability is accompanied not only by a greater heterogeneity of these compound classes but also by their larger concentration. An implication of this dynamics for the amide class is that its resilience to the loss of molecular stability of SOM is concomitant to an enhanced degradation activity of soil microbes [37]. Similarly, a relationship between the heterogeneity and amount of HN in soils with a destabilization of soil organic matter has been already noted [38].

Our results suggest that the molecular stability of humus in soil is a function of nitrogenated or oxygenated aromatic compounds, which are somehow protected
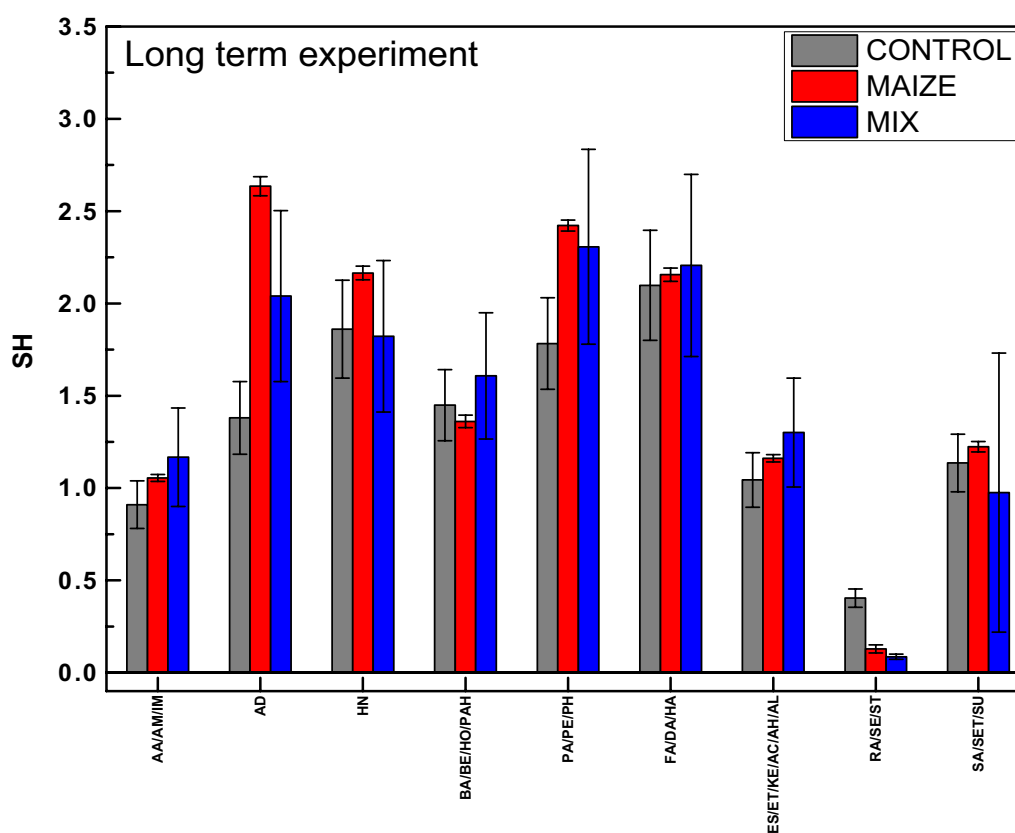
Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 9 of 13

Fig. 3 Shannon Index (SH) derived from nine molecular classes identified by soil humeomic fractionation applied to soils of the 20 years long-term experiment: uncropped (CONTROL), under continuous Maize cultivation (MAIZE), and under Maize–Broad bean rotation (MIX). AA = amino acids; AM = amines; IM = imines; AD = amides; BA = benzoic acids; BE = benzoic esters; HO = heterocyclic oxygen compounds; PAH = polycyclic aromatic hydrocarbons; PA = phenolic acids; PE = phenolic esters; PH = phenols; FA = fatty acids; DA = dicarboxylic acids; HA = hydroxy acids; ES = aliphatic esters; ET = ethers; KE = ketones; AC = alcohols; AH = aldehydes; AL = alkanes/alkenes/alkynes; RA = resin acids; SE = steroids; ST = sterols; SA = sugar acids; SET = sugar esters; SU = sugars; HN = heterocyclic nitrogen compounds

from rapid mineralization. This appears to agree with previous indications which related the recalcitrance of soil organic matter mostly to the Lignin/N and C/N ratios [39]. Furthermore, our findings suggest that, in the case of a Maize short-term cultivation, the molecular diversity in the AD, PA/PE/PH, ES/ET/KE/AC/AH/AL, and HN molecular classes becomes detrimental to the overall soil organic matter stability.

Soil system under long-term cultivation

It has been already reported that the long-term cultivation experiment considered here has revealed the significant loss of hydrophobic molecules with continuous Maize cultivation that was mitigated when a rotation with a leguminous crop was introduced [29]. This trend of progressive reduction in SOM molecular stability is clearly confirmed by the nCPR descriptor derived here from the OC content of the same humeomics fractions, whose values decreased from 0.680 for the uncultivated

control soil, to 0.412 and 0.187 for the MIX and MAIZE soils, respectively.

The values of the Shannon diversity index applied to the nine molecular classes identified by the humeomic fractionation for the soils of the 20 years long-term cultivation experiment are reported in Table 2 and Fig. 3. Summing the single class SH heterogeneity to the total molecular system of each soil humeome, the SH_{tot} increased in the order from the uncropped control soil to those under the MIX and MAIZE treatments. The same was true when the Shannon heterogeneity was weighted for the percent of OC in the different molecular classes ($SH_{tot} \times \% OC$). In fact, the SH_{tot} and the OC weighted SH_{tot} increased, respectively, from 12.064 and 1.323 for the uncultivated control soil, to 13.512 and 1.543 for the Maize–Broad bean rotation, and up to 14.308 and 1.714 for the Maize monocultivation (Table 2). These findings show that the Shannon descriptor can reliably indicate that the progressive loss of molecular homogeneity of the
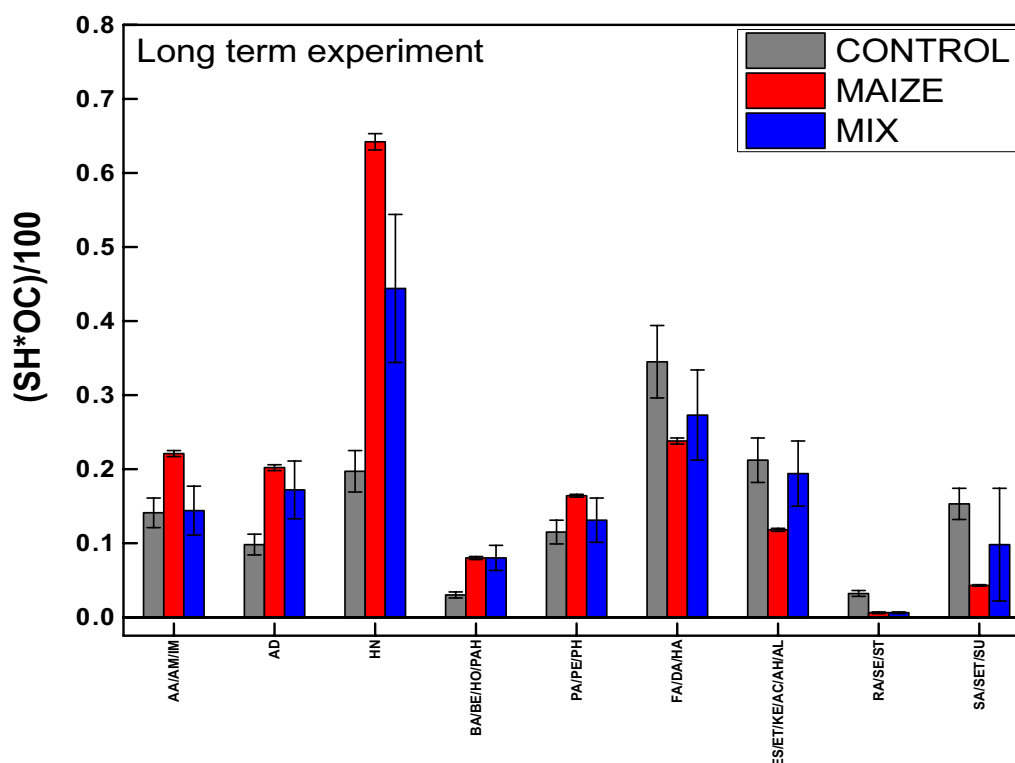
Fig. 4 Shannon (SH) Index weighted for % OC in the nine molecular classes identified by soil humeomic fractionation applied to soils of the 20 years long-term experiment: uncropped (CONTROL), under continuous Maize cultivation (MAIZE), and under Maize-Broad bean rotation (MIX). AA = amino acids; AM = amines; IM = imines; AD = amides; BA = benzoic acids; BE = benzoic esters; HO = heterocyclic oxygen compounds; PAH = polycyclic aromatic hydrocarbons; PA = phenolic acids; PE = phenolic esters; PH = phenols; FA = fatty acids; DA = dicarboxylic acids; HA = hydroxy acids; ES = aliphatic esters; ET = ethers; KE = ketones; AC = alcohols; AH = aldehydes; AL = alkanes/alkenes/alkynes; RA = resin acids; SE = steroids; ST = sterols; SA = sugar acids; SET = sugar esters; SU = sugars; HN = heterocyclic nitrogen compounds

soil humeome is related to an increasing instability of soil organic matter.

Furthermore, the SH values for the molecular heterogeneity in the different soils, can be combined with the nCPR to derive the molecular stability of organic matter ratio (SOMR of Eq. 4), that more directly associates molecular heterogeneity and hydrophobicity of the soil humeome to describe the overall soil organic matter resistance to changes. In fact, the decreasing trend of SOMR values confirmed the loss of molecular stability of SOM in the 20 years long experiment, since it passed from 0.514 of the uncultivated control soil, to the intermediate 0.267 of the partially protected soil humus of the MIX soil, to the least 0.109 of the most exploited soil under maize monoculture (Table 2).

When it was considered the single SH for each of the nine molecular classes identified in the soil humeome of the long-term experiment (Fig. 3), an increasing heterogeneity from CONTROL to MIX and MAIZE soils was noted for AD and HN classes, though only the SH for

amide in the MAIZE soil was significantly different from those of the other two treatments. However, when the SH was weighted for the % OC in the molecular class (Fig. 4), it was only the heterocyclic nitrogen that revealed a substantial difference among soil humeomes, passing from 0.197 of the uncultivated control soil, to 0.444 for MIX soil, and up to 0.642 for that under the MAIZE soil. As in the case of the Maize treatments in the short-term experiment, also after 20 years of cultivation under maize monoculture, the HN compounds increased in heterogeneity and amount in the soil humeome, thus confirming previous observations [40], and suggesting that this class of compounds may well represent a molecular indicator of soil organic matter residual stability.

The relationship between the nCPR and SOMR provided an additional key to assess the dynamics of the molecular stability of soil humus systems in both the short- and long-term experiments. By plotting all SOMR values versus those of nCPR (Fig. 5), it was found that the largest hydrophobicity and homogeneity of the
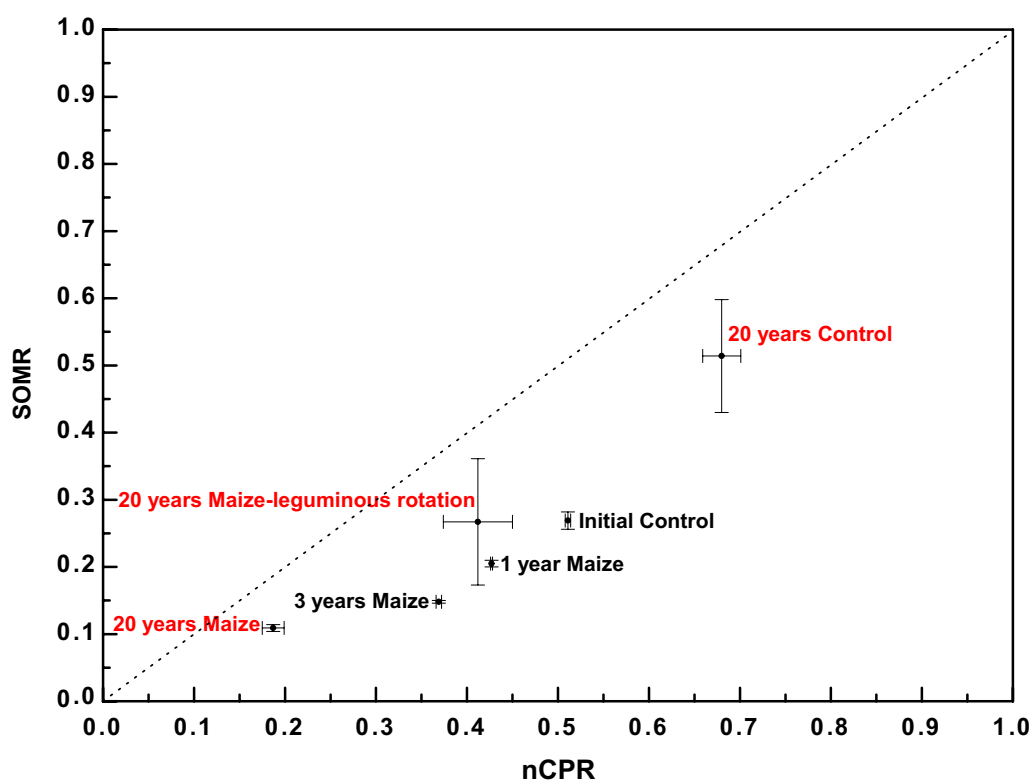
Fig. 5 Plot of SOMR versus nCPR for the soil humeomes of both the short- (black) and long-term (red) experiments

molecular humeome was in the uncropped control soil of the long-term experiment, which also showed the greatest molecular stability of soil humus. Cultivation with Maize decreased significantly both hydrophobicity and molecular resilience of soil humus in both the short- and long-term experiment. Conversely, the MIX treatment in the 20 years long experiment reduced somewhat the loss of hydrophobicity and molecular stability of soil humus, thus revealing SOMR values similar to those of the control soil in the short-term experiment (Fig. 5).

Conclusion

This work showed that using the results of Humeomic fractionation, it was possible to express the heterogeneity of the complex molecular system of soil humus by the simple Shannon descriptor. In fact, the Shannon diversity index was derived from the different components identified in soil humus through the detailed Humeomic chemical sequential fractionation. The Shannon index of molecular heterogeneity applied here to soil humeomes developed under different cropping systems, enabled to also derive an index of humus molecular stability when combined with the degree of hydrophobicity of the soil humeome.

Our findings revealed that heterogeneity and hydrophobicity had opposite trends when soil humus stability

was perturbed by cultivation. In fact, both in the short- and long-term experiments, the hydrophobicity decreased with continuous Maize cultivation, while the heterogeneity of the soil humus molecular system was found to concomitantly enhance. This phenomenon was significantly reduced when a soil management based on a maize-leguminous rotation was adopted, thus confirming the benefits of the rotation practice in maintaining the molecular stability of soil humus, and soil health in general.

The Shannon index derived for specific molecular classes present in the soil humeome also revealed that nitrogenated and/or oxygenated aromatic compounds were mostly responsible for the molecular resilience of soil organic matter. In fact, the short-term experiment showed a decrease of the Shannon diversity index for the classes of amides, phenolic esters/phenolic ethers/phenols, and heterocyclic nitrogen compounds, while the heterogeneity and quantity of heterocyclic nitrogen was related to the overall loss of soil humus molecular stability in the long-term experiment.

The results of this study suggest that the collection of detailed molecular information on the composition of a soil humeome is essential to derive a single numerical descriptor that can accurately highlight the differences in soil humus molecular stability under different land use

Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 12 of 13

and management. Moreover, this descriptor allows calculations of SOMR and nCPR values, which can be used, as in Fig. 5 of this study, to compare different soil types and agricultural practices and serve as a tool for decision makers to program measures of soil reclamation and improvement of soil biodiversity.

Abbreviations

AA	Amino acids
AC	Alcohols
AD	Amides
AH	Aldehydes
AL	Alkanes/alkenes/alkynes
AM	Amines
AQU2	Weakly ester-bound humeomic hydrosoluble fraction
AQU3	Strongly ester-bound humeomic hydrosoluble fraction
AQU4	Strongly ether-bound humeomic hydrosoluble fraction
BA	Benzoic acids
BE	Benzoic esters
CPR	Chemical protection ratio
DA	Dicarboxylic acids
ES	Aliphatic esters
ET	Aliphatic ethers
FA	Fatty acids
HA	Hydroxy acids
HN	Heterocyclic nitrogen compounds
HO	Heterocyclic oxygen compounds
IM	Imines
KE	Ketones
MAIZE	Soil after 20 years of Maize monoculture
MAIZE 1	Soil after 1 year of Maize cultivation
MAIZE 3	Soil after 3 years of Maize cultivation
MIX	Soil after 20 years of maize-broad bean *Vicia faba* L., rotation
nCPR	Normalized chemical protection ratio
OC	Organic carbon
ORG1	Unbound organosoluble humeomic fraction
ORG2	Weakly ester-bound humeomic organosoluble fraction
ORG3	Strongly ester-bound humeomic organosoluble fraction
PA	Phenolic acids
PAH	Polycyclic aromatic hydrocarbons
PE	Phenolic esters
PH	Phenols
RA	Resin acids
RESOM	Final residual soil humeomic hydrosoluble fraction
RESORG	Final residual soil humeomic organosoluble fraction
SA	Sugar acids
SE	Steroids
SET	Sugar esters
SH	Shannon index
SH_{tot}	Total Shannon heterogeneity
SOC	Soil organic carbon
SOM	Soil organic matter
SOMR	Stability soil organic matter ratio
ST	Sterols
SU	Sugars

Author contributions
M.D. and M.O. and A.P. wrote the manuscript. V.C. and A.S. were major contributors in writing the manuscript. M.D., A.S. and A.P. conceptualized and supervised the current article formation. All authors read and approved the final manuscript.

Availability of data and materials
The original data which were analyzed and used to generate the results of this meta-analysis study are included in these published articles [and their supplementary information files]: (1) Drosos M, Nebbioso A, Mazzei P, Vinci G, Spaccini R, Piccolo A. A molecular zoom into soil Humeome by a direct sequential chemical fractionation of soil. Sci Total Environ. 2017;586:807–16. https://doi.org/10.1016/j.scitotenv.2017.02.059. (2) Drosos M, Piccolo A. The molecular dynamics of soil humus as a function of tillage. L Degrad Dev. 2018;29:1792–805. https://doi.org/10.1002/ldr.2989. (3) Savarese C, Drosos M, Spaccini R, Cozzolino V, Piccolo A. Molecular characterization of soil organic matter and its extractable humic fraction from long-term field experiments under different cropping systems. Geoderma. 2021;383:114700. https://doi.org/10.1016/j.geoderma.2020.114700.

Declarations

Ethics approval and consent to participate
This manuscript is an original paper that has not been published in any other journal. The authors agreed to maintain the copyright rule.

Consent for publication
The authors agree with the publication of this manuscript in this journal.

Competing interests
The authors declare that they have no competing interests.

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Drosos *et al. Chem. Biol. Technol. Agric.*   (2023) 10:105

Page 13 of 13

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