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Spectroscopic and Biological Properties of Humic Substances Extracted from Earthworm Coprolites in a Long-Term Experiment Treated with Manure and Mineral Fertilization

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ABSTRACT

Humic substances (HS) are the most important natural soil conditioners because they play an important role in guaranteeing long-term fertility. In this study, the structure and biological properties of HS of earthworm (*Allobophora rosea*) faeces were investigated in order to better understand how fertilization practices can influence their chemical and biological properties. The study was conducted in the Experimental Farm of Padua University, at Legnaro (NE Italy 45° 21' N; 11° 58' E; 6 m a.s.l.) as part of the longest running rotation experiment in Italy. The soil is a fluvi-calcaric cambisol (CMcf), silty or sandy loam, with a sub-basic pH. The trial considers 4 treatments with maize as the main crop. These allow the comparison between fertilization with only organic (farmyard manure, 60 t ha^{-1} y^{-1}, 20% d.m.), only mineral (high mineral input, 300 kg N ha y^{-1}, 66 kg P ha y^{-1}, 348 kg K ha y^{-1}) or mixed inputs (farmyard manure, 30 t ha^{-1} y^{-1}, 20% d.m. plus mineral input, 150 kg N ha y^{-1}, 33 kg P ha y^{-1}, 174 kg K ha y^{-1}) and no fertilization. The experimental layout is a randomized block with three replicates, on plots of 7.8 × 6 m. The coprolites taken from each plot were treated with alkaline solution to extract the HS. Gel permeation chromatography, infrared spectroscopy and surface-enhanced Raman scattering (SERS) were applied to study the structure of HS. The biological activity of HS was also investigated by assaying the auxin and gibberellic-like activity. The results obtained have shown that a relationship exists between different fertilization practices and chemical and biological properties of HS produced by earthworms.

Keywords: auxin-like activity, earthworm coprolites, gel permeation chromatography, gibberellic-like activity, IR, SERS
Abbreviations: HS, humic substances; **SERS**, surface-enhanced Raman scattering

INTRODUCTION

Soil organic carbon (SOC), the most important parameter for long-term studies, is an indicator of soil quality and agronomic sustainability because of its impact on the other physical, chemical and biological indicators of soil quality (Reeves 1997). According to Robinson *et al.* (1996), SOC and its evolution into humic carbon (HC) is arguably the best single indicator of soil quality.

Intensive agronomic practices have a great impact on SOC characteristics, often leading to a decrease in the levels of organic carbon and, as a consequence, to the deterioration of soil quality. In order to maintain agriculture sustainable in the long term, it is necessary to reach a balance between inputs and outputs of soil carbon. Different fertilization practices (farmyard manure, mineral, mixed inputs and no fertilization) influence the content, quality and evolution of soil organic matter (SOM) and an important contribution to improve its content is represented by earthworm faeces.

Earthworms, one of the major macroinvertebrate groups and ecosystem engineers in temperate and tropical soils, have been shown to exert a vital role in the humification of organic matter in natural soils (Zech *et al.* 1997; Feller *et al.* 2003) and in composts (Plaza *et al.* 2008).

In agro-ecosystems, the activity of earthworms strongly depends on management practices such as tillage (Chan 2001), fertilization (Xiang *et al.* 2006), use of organic inputs (Eriksen-Hamel *et al.* 2009), residues handling (Fonte *et al.* 2009) and cropping systems (Simonsen *et al.* 2010).

In the field, the presence of SOM contributes to an increase in local earthworm populations (Huerta *et al.* 2007; Birkhofer *et al.* 2008). When present, earthworms create biogenic aggregates that may influence microbial activity and diversity as well as SOM composition, SOC sequestration and mineralization (Lavelle and Spain 2001; Nguyen *et al.* 2011).

A significant effect of their activities is through the humification process. By grinding and mixing mineral constituents and organic wastes with substances secreted by the gut, earthworms speed up the humification of SOM and stimulate its biological activity (Andersen 1983; Lee 1985). It is known that microbial communities in soil, particularly in the rhizosphere, possess great potential to produce a vast range of metabolites (biologically active substances) that may affect plant growth directly after being taken-up by the plant, or indirectly by modifying the soil environment. These biologically active substances are constituted by the phytohormones (Frankenberger and Arshad 1995). Muscolo *et al.* (1999) reported that the indole-3-acetic acid (IAA) content found in earthworm faeces humic extracts was 7.6 times higher than the soil humic extract where the faeces were collected. This topic deserves particular attention because humic substances (HS) extracted from earthworm faeces have beneficial effects on plant growth with the increase of root growth being generally more apparent than that of the shoot (Nardi *et al.* 1994). Moreover, the stimulatory effects of HS have been correlated to an increase in nitrate metabolism (Muscolo *et al.* 1999). This effect has been mainly attributed to the auxin-like activity of HS and

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also to their chemical composition as evidenced by ^{13}CNMR spectra (Nardi *et al.* 2000). In particular, these analyses demonstrated that the effectiveness of HS on plant metabolism is related to carbohydrate and aliphatic C humic content (Nardi *et al.* 2002).

Other studies showed that two fractions of earthworm HS, differing in nominal molecular weight, containing a very low amount of free IAA, and exhibiting auxin-like properties, caused stomatal opening in the epidermal peels of Argenteum mutant (*Pisum sativum* L.) leaves.

The maximal stomatal apertures in response to both HS were similar to that caused by IAA, confirming that stomatal opening is in response to auxin and HS and involves the activation of a phospholipase A2, which is a component of auxin signaling (Russell *et al.* 2006).

A recent research (Muscolo *et al.* 2012) studied the biological behavior of the HS free from water-soluble phenols (HS-WP) and water-soluble phenols free from humic substances (WP-HS) on different plant organs. The results showed that exist a relationship between chemical composition of humic matter and phenolic compounds and plant biological activity.

The influence of organic amendments on composition of earthworm faeces HS has not been extensively studied. The present study investigated the long-term effect of manure and mineral fertilization on the spectroscopic and biological features of HS from endogenic earthworm faeces.

MATERIALS AND METHODS

Experiment description

A long-term experiment has been underway since 1962 on the Experimental Farm of Padova University, at Legnaro (NE Italy 45°21'N; 11°58'E; 6 m a.s.l.). It is the longest running rotation experiment in Italy. The long term experiments have the purpose to study the turnover of organic matter and the carbon stock during long period.

The soil is a fluvi-calcaric cambisol, silty or sandy loam, with a sub-basic pH. The main physical properties of the top soil layers were reported in a previous paper (Nardi *et al.* 2004). The trial considers four treatments with maize as the main crop. These allow the comparison between fertilization with only organic (L2 = farmyard manure, 60 t ha^{-1} y^{-1}, 20% d.m.; only mineral (M2 = high mineral input, 300 kg N ha y^{-1}, 66 kg P ha y^{-1}, 348 kg K ha y^{-1}) or mixed inputs (L1M1 = farmyard manure, 30 t ha^{-1} y^{-1}, 20% d.m. + mineral input, 150 kg N ha y^{-1}, 33 kg P ha y^{-1}, 174 kg K ha y^{-1} and no fertilization (0) (Giardini *et al.* 1987, 1999).

The experimental layout is a randomized block with three replicates, on plots of 7.8 × 6 m. The faeces of *Nicodrilus* (=*Allolobophora* (Eisen) = *Aporrectodea* (Oerley)) *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) were collected in March and October from the surface of unamended and fertilized soil at the Experimental Farm. After collection, the faeces were immediately air-dried.

Extraction of faeces HS (20 g) was done with 0.1 N KOH (200 mL). The extract was dialyzed against distilled water with a 14 kDa molecular weight cut-off Visking membrane (Medicell, Liverpool, UK) (Nardi *et al.* 1994), desalted with ion exchange Amberlite IR-120 (H^{+} form) (Stevenson 1994). The dialyzed solution was reduced in volume to about 50 mL, and freeze-dried. From 20 g of faeces, 0.76 g of humic carbon was obtained.

Chemical analyses and apparent molecular weight distribution

The pH was determined in water with a coprolites to water ratio of 1: 2.5. The organic C and N content in extracts were measured using an element analyzer (vario MACRO CNS, Hanau, Germany). The organic matter value was obtained by multiplying the organic carbon percentage by 1.72. The HS molecular-weight distribution was determined by gel filtration and chromatography (Nardi *et al.* 2007). The column calibration was based on previously assessed standard proteins (Kit MS-II, Serva, Heidelberg, Germany) (Dell'Agnola and Ferrari 1971). The apparent molecular weight of the fractions was defined as follows: F1 > 100.000 Da; F2 10.000-100.000 Da; F3 < 10.000. This range of molecular weights has been chosen to compare these results with our data-bank.

ATR/FT-IR analysis

The infrared spectra were recorded using a Nicolet 5700 Thermo-Corporation equipped with a diamond attenuated total reflectance (ATR) accessory (Spectra-Tech, Shelton, CT). All spectra were collected by co-addition of 100 scans at a resolution of 4 cm^{-1} in the range 4000-400 cm^{-1}. A background spectrum was recorded using only the diamond crystal prior to collection of each sample spectrum. The spectra were processed using Grams/386 spectroscopic software (Galactic Industries, Salem, NH, USA).

Surface-Enhanced Raman spectroscopy (SERS)

SERS spectra of SOC were recorded using a silver colloid obtained by reduction with citrate (Lee and Meisel 1982). SOC (1 mg) was dissolved in 10 mL of tri-distilled water. Samples for Raman measurements were prepared by adding 10 mL of the SOC solution to 1 mL of silver colloid, and adjusting the pH to *ca.* 12 with 0.1N NaOH. The SERS spectra were recorded with a Renishaw RM2000 Raman spectrometer (Gloucestershire, UK). The 514.5 nm excitation line provided by an Ar^{+} laser was exploited. For measurement, the samples were stored in a quartz cell with 1 cm optical path length placed in a macro-sampling accessory with a focalization lens of 15 mm. The laser power on the sample was 2 mW. The resolution was set at 2 cm^{-1} and the geometry of Raman measurements was 180° (Sànchez-Cortés *et al.* 2006). Normal Raman and SERS spectra were baselined to withdraw the contribution of the fluorescence by using the algorithm provided by the Origin 6.0 Program.

Bioassays

The HS biological activity was assessed by checking the growth reduction of watercress (*Lepidium sativum* L.) roots and the increase in the length of lettuce (*Lactuca sativa* L.) shoots (Audus 1972).

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 10 seeds were aseptically placed on filter paper contained in a Petri dish. For watercress, the filter paper was wetted with 1.2 mL of 1 mM $CaSO_4$ (control); or 1.2 mL of 0.1 and1 mg L^{-1} IAA (Sigma-Aldrich, St. Louis, MO) to obtain the calibration curve; or 1.2 mg L^{-1} of a serial dilution of the products into 1 mM $CaSO_4$. For lettuce, the experimental design was the same as for watercress except that the sterile filter paper was wetted with 1.4 mL of the above solutions and the calibration curve was a progression of 0, 0.1 and 1 mg L^{-1} gibberellic acid (GA) (Sigma-Aldrich, St. Louis, MO).

The seeds were germinated in the dark at 25°C. After 48 h for watercress and 72 h for lettuce, the seedlings were removed and the root or shoot lengths were measured.

Statistical analyses

All data were the means of three measurements for each trial, and the standard deviations did not exceed 5%. Results were processed statistically with the Student-Newman-Keul's test. All statistics were performed by using SPSS for Windows software, version 18.0 (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Chemical composition and apparent molecular weight

According to Satchell (1983) many earthworms require calcium rich foods. As such food derives from vegetables grown on calcitic substrate, it is easy to explain why the number of earthworms is lower in acid soils and higher in calcareous ones. Among physical and chemical properties, the pH regulated earthworm density; a basic pH increased

Table 1A pH, carbon (C), organic matter (OM), humic carbon (HC), nitrogen (N), C/N of coprolites collected in March in soils fertilized with no inputs (0), manure (L2), mineral fertilizers (M2) and with manure plus mineral fertilizers (L1M1).

March	pH	C	OM	HC	N	C/N
Practices		%	%	%	%	
0	8.34 ± 0.02 b	8.06 ± 0.01 c	13.86 ± 0.04 c	0.17 ± 0.02 c	0.89 ± 0.02 c	9.05 ± 0.04 c
L2	8.39 ± 0.02 a	16.47 ± 0.03 a	28.32 ± 0.03 a	0.41 ± 0.02 a	1.46 ± 0.01 a	11.28 ± 0.07 b
M2	8.22 ± 0.01 c	14.10 ± 0.04 b	24.25 ± 0.04 b	0.21 ± 0.01 b	1.11 ± 0.02 b	12.70 ± 0.06 a
L1M1	8.22 ± 0.04 c	17.36 ± 0.01 a	29.8 5± 0.04 b	0.24 ± 0.02 b	1.57 ± 0.02 a	11.05 ± 0.07 b

Data represent the means of three measurements. Values in the same column following the same letter are not statistically different at P<0.05 according to Student-Newman-Keul's test. § = mean ± standard deviation (n = 3)

Table 1B Molecular weight of humic fractions content of coprolites collected in March in soils fertilized with no inputs (0), manure (L2), mineral fertilizers (M2) and with manure plus mineral fertilizers (L1M1).

March	FI	FII	FIII
Practices		%	
0	17.73 ± 0.11 d	63.21 ± 0.12 a	19.06 ± 0.07 a
L2	33.25 ± 0.12 a	56.78 ± 0.02 b	9.97 ± 0.01 c
M2	25.55 ± 0.10 c	62.16 ± 0.13 a	12.26 ± 0.01 b
L1M1	27.63 ± 0.10 b	61.30 ± 0.03 ab	11.07 ± 0.11 b

Data represent the means of three measurements. Values in the same column following the same letter are not statistically different at P<0.05 according to Student-Newman-Keul's test § = mean ± standard deviation (n = 3)

Table 2A pH, carbon (C), organic matter (OM), humic carbon (HC), nitrogen (N), C/N and molecular weight of humic fractions content of coprolites collected in October in soils fertilized with no inputs (0), manure (L2), mineral fertilizers (M2) and with manure plus mineral fertilizers (L1M1).

October	pH	C	OM	HC	N
Practices		%	%	%	%
0	7.72 ± 0.01 a	8.29 ± 0.03 d	19.42 ± 0.03 c	0.30 ± 0.01 b	0.46 ± 0.02 b
L2	7.67 ± 0.03 a	15.58 ± 0.01 a	28.52 ± 0.02 a	0.36 ± 0.03 a	0.71 ± 0.02 a
M2	7.74 ± 0.12 a	12.74 ± 0.02 c	21.91 ± 0.02 b	0.23 ± 0.02 c	0.43 ± 0.01 b
L1M1	7.64 ± 0.11 a	14.78 ± 0.02 b	25.42 ± 0.03 b	0.27 ± 0.01 c	0.61 ± 0.01 a

Data represent the means of three measurements. Values in the same column following the same letter are not statistically different at P<0.05 according to Student-Newman-Keul's test § = mean ± standard deviation (n = 3)

Table 2B Molecular weight of humic fractions content of coprolites collected in October in soils fertilized with no inputs (0), manure (L2), mineral fertilizers (M2) and with manure plus mineral fertilizers (L1M1).

October	FI	FII	FIII
Practices		%	
0	16.56 ± 0.06 c	68.33 ± 0.12 b	15.11 ± 0.10 a
L2	24.78 ± 0.05 a	65.12 ± 1.15 c	10.10 ± 0.03 c
M2	19.51 ± 0.01 b	70.00 ± 1.23 a	10.49 ± 0.13 b
L1M1	24.63 ± 0.13 a	65.11 ± 1.32 c	10.26 ± 0.09 c

Data represent the means of three measurements. Values in the same column following the same letter are not statistically different at P<0.05 according to Student-Newman-Keul's test § = mean ± standard deviation (n = 3)

earthworm multiplication, development and diffusion. On the contrary, at low pH the relative number of earthworms declined in comparison with that of the total fauna in the soil. In the light of this, and considering the sub-alkalinity of soils, the coprolites collected in March had an alkaline pH (**Table 1A**) with values ranging from 8.22, in the sample treated with mineral fertilization (M1), to 8.39 in coprolites taken from soil with manure (L2). In coprolites collected in October (**Table 2A**), the pH remained sub-alkaline in all treatments and no statistical difference was observed among them. However, the pH values estimated were statistically different from those of coprolites collected in March. The difference may be due to the rainfall during the summer that affected the composition of coprolites; in fact Dell'Agnola and Nardi (1987) reported that temperature and humidity are important factors in influencing not only the earthworm biomass but also the chemical characteristic of the faeces.

The evolution of organic matter (OM) in soils is controlled by two processes: mineralization and humification. These processes are regulated by the activity of microorganisms and by the physical and chemical conditions of the soil. The turnover of OM is in relation to the C/N ratio, which is an index of the rate of transformation of the OM to humus. This ratio is high at the beginning of the process to promote the biodegradation of OM and lignin, and approaches the value of 10 at the end of the humification process (Pizzeghello *et al.* 2001).

In both March and October samples, the C and N content showed a considerable increase in all treatments with respect to the untreated soil. In particular, coprolites from L2 and L1M1 led to an enrichment in C and N (**Tables 1A, 2A**). The accumulation of N is very important because it is one of the most important indicators of the soil quality and its quantity is related to production and environmental aspects. The quantity of humic carbon (HC) also increased with respect to the control and as expected, the treatment with L2 led to highest amount of HC (**Tables 1A, 2A**).

The OM amount, following the same trend as organic carbon, evidenced the role of manure in maintaining its content stable; while the mineral fertilizers caused a statistically significant decrease of 10%. The presence of earthworms promoted the release of nitrogenous nutrients and solubilization of phosphate (Mackay *et al.* 1986), therefore, their effects were not limited to mineralization of the organic substrate (Petersen and Luxton 1982), but contributed to the formation of humic matter that mobilized soil nutrients and increased plant metabolism. For these reasons plants have an enhanced absorption capacity and nutrient uptake, and this clearly affects growth and productivity (McColl *et al.* 1982).

The C/N ratio statistically increased in all treatments with respect to the untreated soil in both March and October. The lack of organic and mineral input determined a low C/N ratio (9.0 and 18.02); this might be the consequence of carbon losses through mineralization and the nitrogen maintenance in cultivated soil. In March the C/N ratio was 9.05 in control soil, 12.70 in the soil treated with mineral fertilizers and 11.28 in soil amended with manure, while in coprolites collected in October, the ratios generally increased: 21.94 for soil amended with manure and 29.62 in the soil with chemical fertilizers.

The C/N ratios in the samples collected in March and October had values higher than the control. This trend was particular clear in October coprolites, probably due to the major uptake of N by plants during the summer. Among the treatments, the highest value of the C/N ratio was measured in M2 as the mineral fertilization did not facilitate the evolution of OM in the soil. This occurred as the organic fertilizers are a nutrient source for the plants that may have a stimulating effect on earthworm activity, while the mineral fertilizers limited the earthworms' biomass (Edwards and Lofty 1982).

The molecular weight distribution is reported in **Tables 1B** and **2B**. The HS fraction (FI, > 100 kDa) from coprolites collected in March showed a different percentage in relation to treatments. Manure amendment led to a higher percentage (33.2%) of FI with respect to the other treatments. This suggests that the addition of manure gave the soil high levels of macromolecules, like alkylic and aromatic compounds, that can be easily accumulated and included in the HC structure (Zech *et al.* 1997), e.g. through hydrophobic interaction (Piccolo 2001). In addition, the manure promotes microbial activity and degradation processes of readily biodegradable OM and as a consequence the pathway of humification processes can be favored (Frankenberger and Arshad 1995).

The majority of molecular weight distribution was always in the intermediate fraction (FII). It did not statistically differ between treatments except for L2 treatment. This fraction might also be formed by large aliphatic molecules derived from fresh-carbon input that could easily to be degraded by an increase in microbial activity due to fertilizations (Lugato *et al.* 2008).

The lowest fraction (FIII), typical of compounds that have not been yet undergone the polycondensation process (Dell'Agnola and Nardi 1979), was also influenced by the treatments. The highest percentage was found with no organic or mineral input (control) while the lowest value was determined in L2 treatment. The decrease of FIII in favor of FII and FI could be explained by the fact that the manure in combination with earthworms activity plays an important part in the darkening of mineral soils (melanization) and in the build-up of humic material (Muscolo *et al.* 2009).

The HS molecular weight distribution from coprolites collected in October evidenced a humification pattern similar to that found in March, where the FI is 16.5% in the control, 24.7% in L2 and 19.5% M2. The second fraction represents 68.3% in the control, 65% in L2 and 70% in M2, while the third fraction is 15% in the control and 10% in treated samples.

Our data confirm that the different treatments also influenced the HC molecular complexity. Farmyard fertilizations improved the production of humus with a high degree of polycondensation, a fraction usually linked to soil fertility; the absence of organic fertilizer inputs determined the opposite, with a higher percentage of non-complex and light-weight humus (Nardi *et al.* 2004).

Spectroscopic characterization

The ATR/FT-IR spectra of coprolites were investigated in more detail in the region between 2000-600 cm^{-1} (**Figs. 1, 2**). The changes observed in this region are usually very sensitive to effects of fertilization practices (Ferrari *et al.* 2011; Simonetti *et al.* 2012). In general, the spectra displayed a similar peaks pattern but changed the relative intensity of main bands in relation to treatments. All spectra were characterized by a shoulder at 1711 cm^{-1} due to C=O stretching of COOH and other carbonyl groups. The bands at around 1650–1600 cm^{-1} are generally attributed to several group vibrations including aromatic C=C, C=O stretching of amide I groups (in protein like compounds), quinonic C=O and/or C=O of H-bonded conjugated ketones. The absorption at about 1539 cm^{-1} is preferentially ascribed to stretching of amide II group. The presence of the band at 1512 cm^{-1} indicates the existence of C=C stretching vibrations of aromatic moieties in lignin (Brunow 2001). The bands appearing at 1460-1450 cm^{-1} are generated by C-H deformations and aromatic ring vibrations. The peak at 1420-1390 cm^{-1} is attributed to O-H deformation, C-O stretching of phenolic OH and C-H deformation of CH_2 and CH_3 groups. The bands at about 1378-1330 cm^{-1} are due to aromatic primary and secondary amines. A shoulder at 1260 and a broad band centered at 1216 cm^{-1} are generally ascribed to C-O stretching and O-H deformation of carboxyls and C-O stretching of aryl ethers. In addition, the shoulder at 1260 cm^{-1} might also be due to guaiacyl ring breathing (Francioso *et al.* 2002). A strong band between 1120 and 980 cm^{-1} with a sharp peak centered near 1028 cm^{-1} is due to C-O stretching of polysaccharide or polysaccharide-like substances.

The spectrum of coprolites HS treated with mineral fertilization collected in March (**Fig. 1**) did not differ from that of the control. These changes tended to be more marked with applications of manure (L1M1, L2). In fact, with the manure amendment the HS spectrum showed a considerable increase in carboxyl groups as indicated by the appearance of a new shoulder at 1594 cm^{-1} (COO^- asymmetric stretch) and by the enhancement of the relative intensity of the bands at 1417 (COO^- symmetric stretch) and 1211 cm^{-1} (C-OH stretch). In addition, amide II group vibration at 1538 cm^{-1} decreased with respect to untreated and mineral fertilization. The enhancement in aromatic moieties of lignin derivative (1512 cm^{-1}) might be ascribed to a positive impact of manure on the degradation process of plant residues (Osono and Takeda 2001; Thevenot *et al.* 2010). Instead, L1M1 led to a slight increase of the band assigned to vibration of amide II (1539 cm^{-1}) with respect to other treatments. The mineral input in addition to the manure seemed to preserve the organic N by a mineralization process. This was also supported by N content (**Table 1A**). In brief, the soil amended over 40 years with manure enriched HS from coprolites with aromatic and carboxylic groups than other treatments.

In October the mineral fertilization led to a decrease in relative intensity of stretching vibrations of the band at 1710 cm^{-1} and 1212 cm^{-1} with respect to the control (**Fig. 2**). Moreover, the spectrum changed for the aromatic moieties of lignin derivative collected in March. Manure amendment did not differ from that collected in March but it was more enriched in carboxyl and aromatic groups. Similarity L1M1 treatment induced an increase in carboxyl and carbonyl groups with respect to the other treatments and season.

SERS spectra of coprolites are shown in **Figs. 3** and **4**. The spectra are highly similar showing two main bands at 1611 and 1306 cm^{-1}, which can be assigned to the polycyclic aromatic compounds that are present in the structure of HS (Corrado *et al.* 2008). Associated to these intense bands the band at 737 cm^{-1} appeared at low frequency. It has been assigned to in-plane stretching band of aromatic compounds (Carletti *et al.* 2010). The nature of polycyclic aromatic compounds is still unknown; however, they might originate from human activities (Stevenson 1994). The aromatic compounds are mainly located in inner parts of the macromolecules, in fact these bands can only be seen at alkaline pH due to a partial opening of the three-dimensional structure after breaking the H-bonds that maintain the molecule stable (Corrado *et al.* 2008; Roldan *et al.* 2011). The different intensity of these main bands observed in HS from treated soil can be related to the number of H-bonds existing in the HS structure (Francioso *et al.* 2005). For ins-

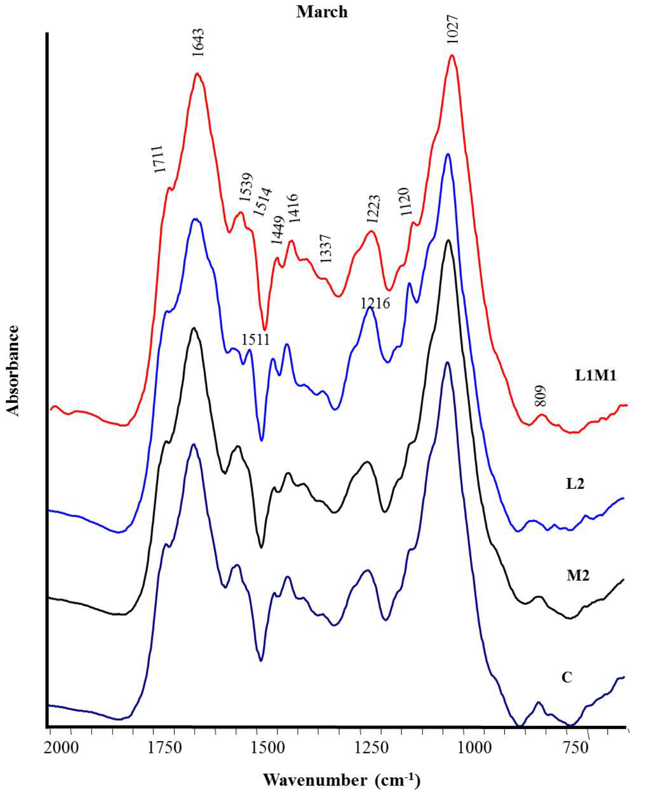

Fig. 1 ATR/FT-IR spectra of HS coprolites (March) extracted from soil treated over 40 years with mineral fertilizer (M2), manure (L2), manure plus mineral (L1M1) and untreated (C).

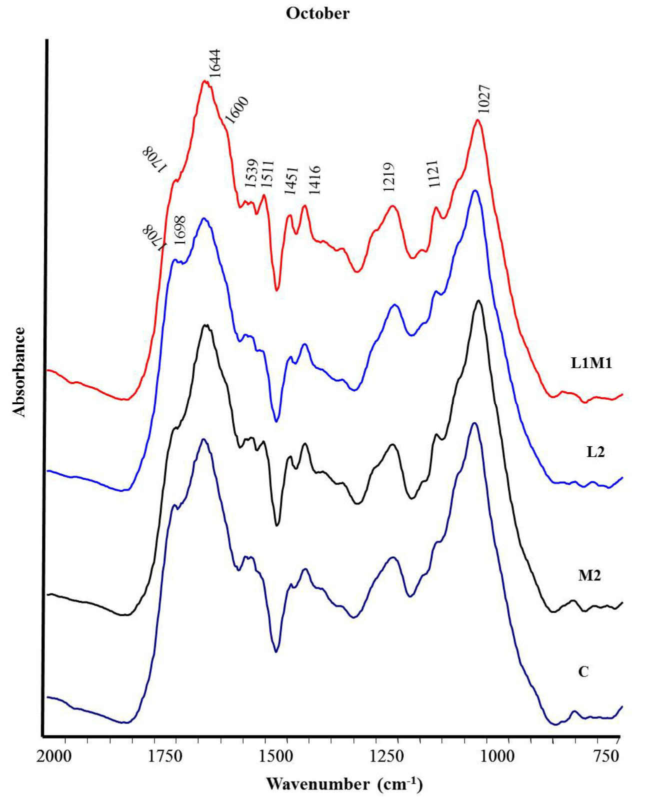

Fig. 2 ATR/FT-IR spectra of HS coprolites (October) extracted from soil treated over 40 years with mineral fertilizer (M2), manure (L2), manure plus mineral (L1M1) and untreated (C).

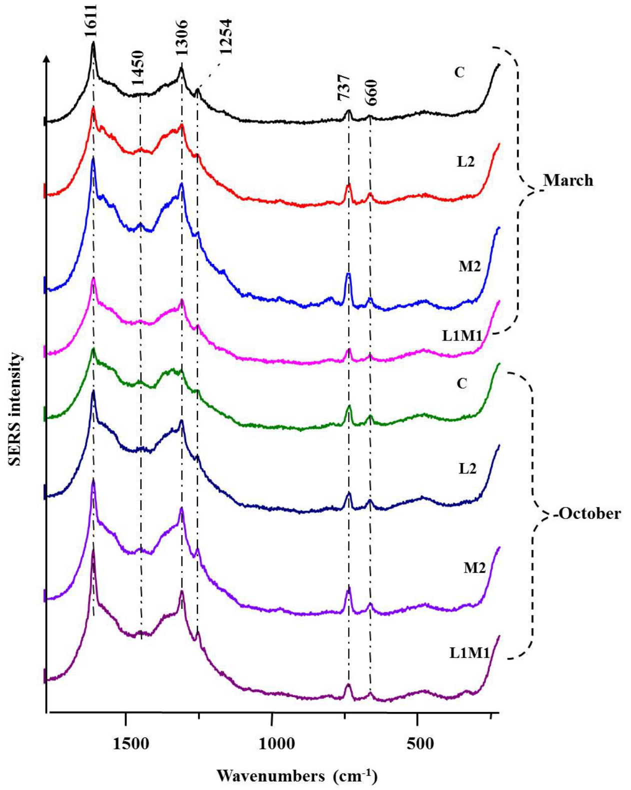

Fig. 3 SERS spectra of HS coprolites extracted from soil treated over 40 years with mineral fertilizer (M2), manure (L2), manure plus mineral (L1M1) and untreated (C).

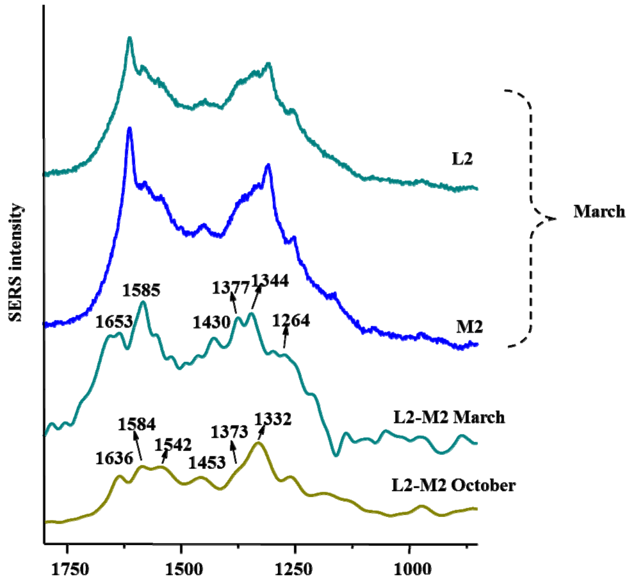

Fig. 4 SERS spectra of HS coprolites extracted from soil treated over 40 years with manure (L2) and mineral (M2) fertilization (up) collected in March. Difference spectra (bottom) were obtained between the spectra corresponding to the samples extracted in March (upper spectra) and those extracted in October (see **Fig. 3**).

tance, in the coprolites HS spectrum of soil amended with manure (L2) (**Fig. 3**) these bands were less intense than in the SERS spectrum from HS from untreated soil (**Fig. 3**) due to the larger amount of H-bonds existing in these compounds. The H-bonds are probably formed as a consequence of continuous input of fatty acids, due to the amendment with manure. The enrichment in fatty acids also leads to a higher stabilization of the macromolecule due to the formation of hydrophobic interactions between aliphatic chains of fatty acid residues (Corrado *et al.* 2008). Other weaker bands also present in the SERS spectra are more difficult to monitor so a further analysis based on difference spectra is required.

To better monitor the influence of manure and mineral fertilization on HS coprolites the difference spectra between the spectra corresponding to these samples were obtained.

Fig. 4 shows the L-M difference spectra for the samples extracted in March (L2, M2) and October (L2, M2).

The difference spectra from samples collected in March showed positive bands at 1585 and 1344 cm^{-1} that are associated to ν(C=C) and ν(C-O) vibrations in polyphenols (Corrado *et al.* 2008; Roldan *et al.* 2011). Furthermore, the bands appearing at 1377 and 1430 cm^{-1} can be assigned to $\nu_s(COO^-)$ in aromatic moieties and $\delta(CH_2)$ in aliphatic moieties. Moreover, the band at 1653 cm^{-1} can be attributed to ν(C=O) in aromatic ketones, while the difference band seen at 1264 cm^{-1} is attributed to ν(C-O) vibrations in polyphenolic moieties.

In the case of the difference spectra from samples collected in October, L2-M2 spectrum showed the most intense bands at 1577 and 1332 cm^{-1}, due to polyphenols vibrations. The positive band at 1636 cm^{-1} is due to ν(C=O) in aromatic ketones, while that at 1453 cm^{-1} is attributed to $\delta(CH_2)$. In this case a strong difference band is seen at 1542 cm^{-1}. This band can also be attributed to polyphenolic groups with a high contribution from guaiacol moieties as also supported by the IR spectrum. The increase of carboxylic groups can also be evidenced by a slight positive shoulder at 1377 cm^{-1}. Thus, the treatment with manure induced an increase of polyphenolic compounds in HS coprolites in comparison to the mineral fertilization. In addition manure amendment increased the carboxylate, ketones and hydroxyl groups as compared to the mineral fertilization.

Bioassays

The Audus (1972) test was used in order to establish the hormone-like activity of HS coprolites. With respect to the IAA-like activity, the increasing concentration of pure indoleacetic acid strongly reduced the root length of watercress (Sabatini *et al.* 1999). This trend was in line with a dose-dependent slight reduction of root length evidenced by 0 and L2 HS application. More specifically, the HS extracted from the control soil significantly reduced the watercress growth by 73% at 0.1 mg L^{-1} and 70% at 1 mg L^{-1} (**Table 3**). L2 HS application caused a significant watercress inhibition of 60% at 0.1 mg L^{-1} and 54% at 1 mg L^{-1}. HS obtained from 0 and L1M1 soils, collected in October, significantly reduced the epicotyl length in a dose-dependent manner. In particular, HS extracted from control soil significantly inhibited watercress by 47% at 0.1 mg L^{-1} and 46% at 1 mg L^{-1}, while the treatment with L1M1 caused a significant reduction of 40% at 0.1 mg L^{-1}and 35% at 1 mg L^{-1}.

The induced responses in terms of gibberellin-like activity (**Table 4**) indicated that the lettuce treated with exogenous gibberellic acid (GA) displayed a dose-dependent increase in the epicotyl ratio (Sun and Gubler 2004) in a significant manner.

Similarly, the L2 treatment collected in March induced a positive effect on lettuce. In particular, the HC extracted from control soil caused a significant increase in epicotyl length of 24% at 0.1 mg L^{-1} and 25% at 1 mg L^{-1}. The best values of epicotyl length stimulations (plus 41% at 0.1 mg L^{-1} and plus 48% at 1 mg L^{-1}) were observed in L2 coprolites. Interestingly, it was observed that HS coprolites collected in soil with mineral and manure inputs showed a significant increase of 22% at 0.1 mg L^{-1}and 27% at 1 mg L^{-1}.

In October, only HS coprolites collected in the control soil elicited gibberellin-like activity that caused a statistically significant stimulation of 29% at 0.1 mg L^{-1} and 32% at 1 mg L^{-1}.

During the last 30 years, the biological activities of HS, particularly those derived from earthworm faeces, have begun to be investigated. The research has demonstrated that the stimulation of plant metabolism due to earthworm HS is imputable to hormone-like behavior.

The present study showed that the HS extracted from coprolites collected in untreated soil, in which there were no fertilization inputs, possess hormone-like activity in both sampling periods. From the literature it is known that soils have a different content of "endogenous" IAA and GA (Pizzeghello *et al.* 2001). Generally, the amount of these hormones is higher in the rhizosphere than the "bulk" of the soil, probably as a result of an accelerated microbial metabolism. There are numerous studies that show the ability of microorganisms to produce auxins and gibberellins, but there are few data regarding the stability of these hormones in the soil (Frankenberger and Arshad 1995). Probably, the HS, thanks to their large specific surface area and the high number of functional groups, are able to preserve these molecules with hormone-activity from degradation, as happens in most soils not disturbed by human activities. It is known (Kale 1998) that agronomic practices and the activity of earthworms are closely related; in general, in soils in which the level of disturbance is lower, earthworms are most abundant and the community biomass is higher (Decaëns and Jiménez 2002).

Table 3 Auxin-like activity of humic substances extracted from: 0 = control; L2 = manure; M2 = mineral and L1M1 = manure plus mineral evaluated by measuring the reduction in the root length (mm) of treated watercress.

Treatments	Concentration	Root length (mm)	
	mg L^{-1}	March	October
Control	-	10.24 ± 0.04 a	11.88 ± 0.04 a
IAA	0.1	6.73 ± 0.04 e	6.73 ± 0.02 d
IAA	1	6.21 ± 0.03 f	6.01 ± 0.05 e
0	0.1	7.43 ± 0.02 c	5.59 ± 0.09 f
0	1	7.12 ± 0.02 d	5.42 ± 0.02 g
L2	0.1	6.23 ± 0.04 f	5.10 ± 0.03 h
L2	1	5.52 ± 0.03 h	2.38 ± 0.02 m
M2	0.1	8.20 ± 0.06 b	7.90 ± 0.04 b
M2	1	6.75 ± 0.04 e	7.54 ± 0.04 c
L1M1	0.1	5.08 ± 0.02 i	4.78 ± 0.02 i
L1M1	1	5.95 ± 0.04 g	4.13 ± 0.02 l

Data represent the means of three measurements with ten plants in each. Values in the same column following the same letter are not statistically different at $P<0.05$ according to Student-Newman-Keul's test. § = mean ± standard deviation (n = 30)

Table 4 Gibberellin-like activity of humic substances extracted from: 0 = control; L2 = manure; M2 = mineral and L1M1 = manure plus mineral evaluated by measuring the increase in the shoot length (mm) of treated lettuce.

Treatments	Concentration	Shoot length (mm)	
	mg L^{-1}	March	October
Control	-	13.32 ± 0.06§i	13.03 ± 0.03§l
GA	0.1	18.10 ± 0.06 c	19.60 ± 0.06 a
GA	1	18.34 ± 0.04 b	18.25 ± 0.06 b
0	0.1	16.58 ± 0.05 f	16.76 ± 0.04 e
0	1	16.67 ± 0.02 e	17.23 ± 0.04 d
L2	0.1	18.83 ± 0.02 b	17.79 ± 0.03 c
L2	1	19.72 ± 0.04 a	16.35 ± 0.03 g
M2	0.1	16.65 ± 0.04 e	18.32 ± 0.03 b
M2	1	16.49 ± 0.04 g	16.04 ± 0.03 h
L1M1	0.1	16.31 ± 0.05 h	16.72 ± 0.02 f
L1M1	1	16.93 ± 0.04 d	14.84 ± 0.02 i

Data represent the means of three measurements with ten plants in each. Values in the same column following the same letter are not statistically different at $P<0.05$ according to Student-Newman-Keul's test. § = mean ± standard deviation (n = 30)

However, one of the cropping practices that increased the biological activity was the distribution of manure. It is known that farmyard manure treatments can maintain soil nutrient levels and stimulate various aspects of soil fertility, i.e. farmyard manure ensures the largely constant presence of active microorganisms and the regular dynamics of biomass C (Nannipieri 1993). In particular, farmyard manure directed the turnover of SOM towards the humification process with a high-quality production of HS. Establishing a relationship between structural composition and biological activity of HS is important for the development of biological resources to be applied in modern sustainable agriculture. However, this is no simple task because of the humic molecular complexity, as well as the plethora of plant biochemical processes modified by HS applications.

CONCLUSIONS

In conclusion, our results indicate the existence of a relationship between different fertilization practices and chemical and biological properties of HS produced by earthworms. In particular, the best results in terms of biological activity were obtained in the trial that had received organic manure. As demonstrated by Frankenberger and Arshad (1995), the hormone-like activity of HS is not due to an improved availability of micronutrients, but to a release of bioactive molecules and/or hormones by the soil microorganisms.

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