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Effects of sewage sludge amendment on humic acids and microbiological properties of a semiarid Mediterranean soil

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Abstract The residual effects of adding 40 t ha^{-1} sewage sludge (SL) to a degraded soil cropped with barley were investigated after 9 and 36 months in a field experiment under semiarid conditions. The principal soil properties were apparently still affected by SL amendment 9 months after application but the effects disappeared after 36 months. With respect to control soil humic acids (HAs), the SL-HA was characterized by higher contents of S- and N-containing groups, smaller contents of acidic groups, a prevalent aliphaticity, extended molecular heterogeneity, and smaller degrees of aromatic polycondensation and humification. Amendment with SL caused an increase in N, H, S and aliphaticity contents and a decrease in C/N ratios and O and acidic functional group contents in soil HAs isolated 9 months after SL application. These effects tended to decrease after 36 months, most probably because the slightly humified SL-HA was mineralised over time through extended microbial oxidation, while only the most recalcitrant components such as S-containing and aromatic structures were partially accumulated by incorporation into soil HA. Microbial biomass, basal respiration, metabolic quotient and enzymatic activities increased in soil 9 months after SL application, possibly because of increased soil microbial metabolism and enhanced mineralisation processes. After 36 months these properties returned to values similar to those of the unamended soil, presumably due to the loss of energy sources.

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Introduction

Soil organic matter (SOM) plays an essential role in nutrient (N, P, S, K) cycles, soil stability and the ecological and environmental aspects of sustainability of soil fertility. SOM represents a major pool of C, estimated to be about $1{,}500–1{,}600\times10^{15}$ g, i.e. 2–3 times the atmospheric CO_2 pool, and acts in soil as both a source and a sink for C and nutrients (Bouwman 1990; Rosenberg and Izaurralde 2001). Humic substances, and among these the humic acid (HA) fraction, are universally recognized to be among the most reactive SOM components that contribute substantially to maintaining the global soil fertility status and agricultural production and to protecting the soil from degradation and contamination (Stevenson 1994). Mediterranean soils are often subjected to severe degradation processes accompanied by a decline of the SOM content, which contributes to a loss of soil fertility (Smith et al. 1993).

The application of sewage sludge (SL) to soil has become common practice due to its increased production, the reduction of available disposal sites and its potential to increase soil fertility (Hall 1995). To ensure the safe and efficient use of SL as a soil amendment, it is important to control the content and chemical quality of its organic matter, especially of the HA-like components, which are good indicators of the biological and chemical stability of SL (Senesi et al. 1996). Furthermore, organic matter from the SL amendment has a significant influence on the status of the soil microbial biomass in that it provides a new energy source that directly affects soil microbiological populations and activity. As a consequence, most physical and chemical soil properties and processes, including the decomposition of SOM, nutrient availability, the synthesis of HAs, and the interactions of HAs with inorganic soil components and inorganic and

organic pollutants, will be modified following addition of SL to soil (Clapp et al. 1986, 2001).

Currently, there is a growing interest in developing valuable and sensitive indicators of soil quality which can reflect the effects of land management and ensure long-term sustainability of soil fertility (Bandick and Dick 1999). In this context, a number of biological and biochemical properties can be used as early and sensitive indicators of SOM transformations and dynamics, nutrient cycling, and stress and recovery conditions in soil (Dick and Tabatabai 1993).

Research on the effects of soil amendment with SL has mainly focused on the potential risks of the introduction of heavy metals into the food chain (Barbarick et al. 1997; Logan et al. 1997) and on its action as a soil conditioner (Sastre et al. 1996; Johansson et al. 1999). Limited information is available on the influence of SL amendment on the content and quality of soil HAs in relation to the microbial activity of SL-amended soils.

The objectives of the present study were to evaluate, in a long-term field experiment, the effects of SL amendment on: (1) the compositional, structural and functional characteristics of soil HAs; and (2) some soil microbiological and biochemical parameters including basal respiration, microbial biomass C, metabolic quotient (qCO_2), and enzyme activities involved in oxidation processes and nutrient cycling.

Materials and methods

SL and soil sampling sites

The SL sample used in this work was a mixture of SLs collected from three water treatment plants in the municipal districts of Madrid, Spain. The field experiment was conducted at the experimental farm Los Pinos located in Pinto, Madrid, Spain, on a Calcaric Regosol (FAO-ISSS 1998), a calcareous sandy soil with low SOM and nutrient contents. The site is characterized by a continental semi-arid climate with an average annual rainfall of 399.1 mm and an average annual temperature of 14.6°C.

The experiment included plots cropped with barley (*Hordeum vulgare*), either unamended (control) or amended with a single SL application of 40 t ha^{-1} in September, before barley sowing (mid-October). Approximately 9 and 36 months after SL application, surface (0–20 cm) soil subsamples were collected randomly from each plot after barley harvest (mid-June). Then, a composite soil sample was obtained for each of the three soils (the control soil and the amended soils sampled after 9 and 36 months) by mixing equal amounts of the corresponding subsamples.

Prior to chemical analyses, plant residues and stones were removed from soils, and soil and SL samples were air-dried, crushed and passed through a 2-mm sieve. Soil samples for microbiological and biochemical analyses were stored at 4°C at field moisture.

Soil and SL chemical analyses

The principal properties of soil and SL samples were determined by standard methods (Sparks et al. 1996), and each sample was analysed in triplicate. The pH and electrical conductivity (EC) were measured on a 1:5 sample:water extract after shaking for 30 min. The organic C content was determined by dichromate oxidation and subsequent titration with ferrous ammonium sulphate (Walkley and Black 1934). The total N content was obtained by the Kjeldahl method and spectrophotometric determination by a Bran-Lubbe Technicon autoanalyser AAII (Buffalo Grove, Ill.) (Hinds and Lowe 1980). P was extracted with calcium carbonate and magnesium carbonate solution and its content determined colorimetrically (Burriel and Hernando 1950). The total contents of metals were determined on 0.5-mm sieved samples after nitric and perchloric acid digestion by using a Perkin Elmer (PE) HGA500 atomic absorption spectrometer (Norwalk, Conn.) (Bureau 1982).

HA isolation

Carbonates were removed from soil samples by mechanical stirring with 2 M H_3PO_4 for 30 min (Midwood and Boutton 1998). The treatment was repeated 3 times. The samples were then washed with distilled water until the suspension reached a pH of 7. HAs were isolated from soils and SL by a conventional procedure (Schnitzer 1982). Briefly, carbonate-free soils and the freeze-dried SL sample were extracted with 0.1 M $Na_4P_2O_7$ (pH 9.8) and then with 0.1 M NaOH, at room temperature (RT, about 293 K), using a sample:extractant ratio of 1:10. Each extraction was repeated 3 times. For each extraction step, the mixture was shaken mechanically for 3 h, then centrifuged at 15,300 *g* for 15 min, and the supernatant was filtered through a Whatman no. 31 filter paper. The combined alkaline extracts were then acidified with HCl to pH 1, left standing for 24 h in a refrigerator to allow the complete precipitation of HA, and then centrifuged at 30,100 *g* for 15 min. The HA precipitates were purified by dissolution in 0.1 M NaOH, centrifugation at 30,100 *g*, elimination of the residue, and acidification of the alkaline supernatant with HCl to pH 1. The suspension was left standing for 12 h at RT, and then centrifuged at 30,100 *g* for 15 min. This procedure was repeated 3 times. The precipitated HAs were then recovered with distilled water, dialysed until free of Cl^- ions, and finally freeze-dried.

HA analyses

Moisture and ash contents were measured by heating the HAs for 24 h at 105°C and 550°C, respectively. The elemental composition was determined by a C, H, N, S analyser (Fisons Instruments, Crawley, UK) model EA 1108. O was calculated by difference: O%=100−(C+H+N+S)%. Total acidity and carboxyl group contents were determined according to conventional methods (Schnitzer 1982), and phenolic hydroxyl group content was calculated by difference.

Absorbances at 465 nm and 665 nm were measured using a PE Lambda 15 UV-Vis spectrophotometer on solutions of 3.0 mg of each HA in 10 ml of 0.05 M $NaHCO_3$, with pH adjusted to 8.3 with 0.02 M NaOH (Chen at al. 1977). The ratios of absorbances at 465 nm and 665 nm gave the E_4/E_6 ratio.

Fourier transform infrared (FTIR) spectra of HAs were recorded over the range 4,000–400 cm^{-1} on pellets obtained by pressing under reduced pressure a mixture of 1 mg HA and 400 mg dried KBr (spectrometry grade). A Nicolet (Madison, Wis.) 5PC FTIR spectrophotometer operating with a peak resolution of 2 cm^{-1} and Omnic 1.2 software were used to obtain and analyse the spectra.

Fluorescence spectra in the emission, excitation and synchronous-scan modes were obtained on aqueous solutions of HA at a concentration of 100 mg l^{-1} after overnight equilibration at RT, and adjustment to pH 8 with 0.05 M NaOH. Spectra were recorded using a PE LS-5 luminescence spectrophotometer equipped with a PE data station 3600 for data generation and processing by PE computerized luminescence spectroscopy software. Emission and excitation slits were set at a 5-nm band width, and a scan speed of 120 nm min^{-1} was selected for both monochromators. Emission spectra were recorded over the range 380–550 nm at a constant excitation wavelength of 360 nm. The overall relative fluorescence intensity (RFI) was expressed in arbitrary units as the unitless reciprocal to the gain used to normalize each emission spectrum (Senesi et al. 1991). Excitation spectra were obtained over a scan

range of 300–500 nm by measuring the emission radiation at a fixed wavelength of 520 nm. Synchronous-scan excitation spectra were measured by scanning simultaneously both the excitation (exc; varied 300–550 nm) and the emission (em) wavelengths, while maintaining a constant, optimised wavelength difference $\Delta\lambda=\lambda_{exc}-\lambda_{em}=18$ nm (Senesi et al. 1991).

Soil biological and biochemical analyses

Basal respiration was evaluated according to Stotzky (1965) by trapping CO_2 evolved from 50 g of each soil (at 70% of its field capacity) in 15 ml of 0.1 M NaOH in sealed polyethylene flasks during a 14-day incubation at 28°C. Carbonates were precipitated with 0.375 M $BaCl_2$ and the residual NaOH was titrated with 0.1 M HCl. The microbial biomass C was determined by an extraction method by using ethanol-free $CHCl_3$ for 12 h at 25°C, and subsequent extraction with 0.5 M K_2SO_4 (Vance et al. 1987). The qCO_2 was calculated by dividing the CO_2-C released from the sample in 1 h by the microbial biomass C content.

Dehydrogenase activity was determined using the method of Skujins (1976) as modified by García et al. (1993). Soil (1 g) at 60% of its field capacity was incubated with 0.2 ml of a 0.4% aqueous solution of 2-*p*-iodo-nitrophenyl-phenyltetrazolium chloride for 20 h at 22°C in the dark. The iodo-nitrophenyl formazan produced in the reaction was extracted with 10 ml CH_3OH by shaking vigorously for 1 min and filtering through a Whatman no. 5 filter paper, and then measured spectrophotometrically at 490 nm. Catalase activity was determined by titrating with $KMnO_4$ the residual H_2O_2 added to soil and not degraded by catalase (Rodríguez-Kábana and Truelove 1982). Urease and protease activities were measured as the amount of NH_4^+ released from 0.5 g soil after incubation for 90 min with the substrates 1 M urea at 30°C and 0.03 M *N* α-benzoyl-argininamide (BAA) at 39°C, respectively, in 2 ml of 0.1 M phosphate buffer at pH 7 (Nannipieri et al. 1980). Phosphatase and β-glucosidase activities were measured by spectrophotometrical determination at 398 nm (Tabatabai and Bremner 1969) of the amounts of *p*-nitrophenol released from 0.5 g soil after incubation at 37°C for 90 min with the substrates 0.115 M *p*-nitrophenyl phosphate disodium and 0.05 M *p*-nitrophenyl-β-D-glucopyranoside, respectively, in 2 ml of 0.1 M maleate buffer at pH 6.5 (Masciandaro et al. 1994). The reaction was stopped by adding trishydroxymethyl aminomethane for the β-glucosidase assay (Tabatabai 1982), and by cooling at 2°C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added and the mixture was centrifuged at 2,000 *g* for 5 min.

For each enzyme assay, a blank experiment was performed which consisted of adding the substrate to the soil sample after incubation and immediately prior to stopping the reaction.

Results and discussion

SL and soils

Table 1 shows some properties of the SL, the unamended control soil, and soils amended with 40 t SL ha^{-1} and sampled 9 and 36 months (S9 and S36, respectively) after amendment. The SL sample has a neutral pH, an acceptable EC, amounts of organic C and N and a C/N ratio within the common range found for SL, and a large P content. Total contents of heavy metals are below the limits imposed by the Spanish legislation for SLs (BOE 1990).

The control soil is slightly alkaline possibly due to the high calcium carbonate content, and has a low EC value, low contents of organic C, N, P and K, and trace element contents that are within the common range found in soils (Kabata-Pendias 2001). The amended soil S9 has lower pH (around neutrality) and C/N ratio, and higher EC value, organic C, N, P, K and metal contents than the control soil. These results are an evident consequence of SL application to soil. However, these effects apparently disappear in soil S36 that shows chemical properties very similar to those of the control soil. The apparent marked decrease in Mn, Zn, Cu, and Ni contents measured in the upper layer (0–20 cm) of soil S36 may be ascribed to metal mobilization in the form of soluble complexes with dissolved organic matter (Vulkan et al. 2002). This would result in the apparent loss of metals from the surface soil layer and in their leaching to subsoil and/or underground waters and/or in the possible uptake by barley crops (Hooda et al. 1997; Keller et al. 2002).

Table 1 Some properties (±SEs) of the sewage sludge (*SL*), unamended control soil and SL-amended soils sampled 9 months (*S9*) and 36 months (*S36*) after SL application. *EC* Electrical conductivity

Parameters measured	SL	Control soil[a]	SL-amended soils	
			S9	S36
pH	7.3±0.1	8.1±0.2	7.4±0.1	8.0±0.1
EC (mS cm^{-1})	3.2±0.0	0.2±0.1	1.4±0.1	0.2±0.1
Organic C (g kg^{-1})	172.1±0.2	10.1±0.2	19.0±0.3	10.9±0.1
Total N (g kg^{-1})	36.5±0.2	1.1±0.1	2.5±0.2	1.2±0.1
C/N ratio	4.7±0.1	9.2±0.1	7.6±0.1	9.1±0.1
P (g kg^{-1})	25.60±0.02	0.12±0.01	0.45±0.03	0.19±0.01
K (g kg^{-1})	2.41±0.05	0.24±0.01	0.34±0.01	0.18±0.01
Ca (g kg^{-1})	32.33±0.05	5.02±0.09	6.23±0.13	5.23±0.21
Mg (g kg^{-1})	5.91±0.02	0.26±0.03	0.31±0.01	0.26±0.01
Na (g kg^{-1})	1.62±0.00	0.01±0.00	0.03±0.00	0.02±0.00
Mn (mg kg^{-1})	167.0±0.2	134.0±12.3	244.0±14.7	151.0±1.4
Zn (mg kg^{-1})	560.0±0.3	19.0±2.9	110.0±5.4	22.0±2.4
Cu (mg kg^{-1})	149.0±0.0	3.0±0.2	19.0±0.3	3.0±0.7
Pb (mg kg^{-1})	43.0±0.0	0.4±0.0	0.4±0.0	0.4±0.0
Cd (mg kg^{-1})	2.0±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Cr (mg kg^{-1})	45.0±0.1	0.4±0.0	0.4±0.0	0.4±0.0
Ni (mg kg^{-1})	30.0±0.1	4.0±0.4	7.0±0.5	3.0±0.0

[a] Texture: sand, 41%; silt, 30%; clay, 29%. $CaCO_3$ content, 24%

Table 2 Elemental composition (on moisture and ash-free basis) of humic acids (*HAs*) isolated from SL, control soil, and S9 and S36. For other abbreviations, see Table 1

Origin of HAs	C	H[a]	N[a] %	S[a]	O	C/N[a]	C/H[a]	O/C[a]
SL	58.9±0.6	8.7	6.8	1.2	24.4±0.2	10.2	0.6	0.4
S9	53.9±0.5	6.1	5.6	0.9	33.5±0.5	11.3	0.7	0.4
S36	55.0±0.3	5.3	4.6	0.6	34.5±0.4	14.0	0.8	0.4
Control	55.4±0.7	4.9	4.0	0.4	35.3±0.3	16.3	0.9	0.5

[a] SEs of three laboratory replicates for N, H, and S% and C/N, C/H, and O/C ratios were always <0.1

Table 3 Acidic functional group contents (on moisture and ash-free basis), E_4/E_6 ratio and relative fluorescence intensity (*RFI*) of HAs isolated from SL, control soil, and S9 and S36 . For other abbreviations, see Table 1

Origin of HAs	Total acidity (mmol g^{-1})	COOH (mmol g^{-1})	Phenolic OH (mmol g^{-1})	E_4/E_6 ratio	RFI
SL	3.25	1.30	1.95	4.2	8.6
S9	4.62	2.36	2.25	4.7	10.3
S36	5.88	3.30	2.57	4.7	5.4
Control	6.99	3.48	3.51	4.6	4.4

Humic acids

Elemental and functional group composition, E_4/E_6 ratio

The elemental and functional group composition of SL-HA is markedly different from that of soil HAs (C-HA, S9-HA and S36-HA) (Tables 2, 3). In particular, C, H, N and S contents of SL-HA are higher, and O, total acidity, carboxyl and phenolic OH contents and C/N and C/H ratios are smaller than the corresponding values of the control HAs. These results are in general agreement with those of previous studies of SL-HAs of various origin in comparison to unamended and amended soil HAs (Senesi et al. 1996; Soler-Rovira et al. 2002).

The elemental and functional group composition of S9-HA and S36-HA is generally intermediate between those of the control HA and SL-HA (Tables 2, 3). In particular, SL application causes an increase in N, H and S contents and a decrease in C/N, C/H and O/C ratios, total acidity, COOH and phenolic group contents of soil HA, whereas C and O contents remain almost unchanged. In comparison to control soil HA, the sample S9-HA shows more pronounced compositional differences than does S36-HA. This result indicates that with increasing time after SL application the composition of amended soil HA approaches that of the control soil HA, i.e. SL-HA evolves in soil tending to become similar in composition to native soil HA. Similar results were obtained in a previous study using a pig-slurry as soil amendment (Plaza et al. 2002).

The E_4/E_6 ratio of SL-HA is smaller than those of soil HAs (Table 3), which can be related to its lower acidic functional group content (Chen et al. 1977). However, the E_4/E_6 values are similar for all soil HAs studied.

Infrared spectra

The FTIR spectra of the HAs studied, and especially the spectrum of SL-HA with respect to those of soil HAs, show several important differences (Fig. 1). The main features of these spectra can be comparatively described as follows. An intense broad peak at about 3,300–3,350 cm^{-1} is present in all spectra, and attributed to H-bonded OH and, secondarily, NH stretching of various functional groups. Two peaks occur at about 2,920–2,950 and 2,850 cm^{-1} that are ascribed to the stretching of aliphatic C-H groups, whose relative intensity decreases in the order: SL-HA>S9-HA>S36-HA>C-HA. An intense peak is apparent at 1,716 cm^{-1}, due to the C=O stretching of various carbonyl groups, including COOH, which is evident only in samples C-HA and S36-HA. An intense broad peak occurs in the region between 1,670 and 1,620 cm^{-1}, which is generally considered an envelope of unresolved absorptions mainly due to aromatic C=C, C=O stretching of amide groups (amide I band), quinonic C=O and/or C=O of H-bonded conjugated ketones, which is relatively less intense in sample SL-HA than in soil HAs. A peak or shoulder is present around 1,540 cm^{-1} that is preferentially ascribed to N-H deformation and C=N stretching of amides (amide II band), whose relative intensity decreases in the order: SL-HA>S9-HA>S36-HA>C-HA. A peak at about 1,460 cm^{-1}, attributed to aliphatic C-H, is evident only in sample SL-HA, and appears as a weak shoulder in soil HAs. A peak at about 1,410 cm^{-1}, preferentially assigned to O-H deformation and C-O stretching of phenolic OH, is apparent only in sample SL-HA and is a faint shoulder in soil HAs. A peak at about 1,375–1,380 cm^{-1}, possibly attributed to C-H deformation of CH_2 and CH_3 groups and/or to anti-symmetric stretching of COO^- groups, shows a relative intensity decreasing in the order: SL-HA>S9-HA>S36-HA>C-HA. An intense broad peak at about 1,230–1,220 cm^{-1}, generally ascribed to C-O stretching and O-H deformation of COOH groups and C-O stretching of aryl ethers, has a relative intensity decreasing in the order: C-HA>S36-HA>S9-HA>SL-HA. A faint peak at 1,165 cm^{-1}, attributed to C-O of various alcoholic and ether groups, is present in sample SL-HA and S9-HA. Finally, an intense absorption at about 1,040–1,030 cm^{-1}, generally attributed to C-O stretching of polysaccharides and polysaccharide-like substances, exhibits a relative

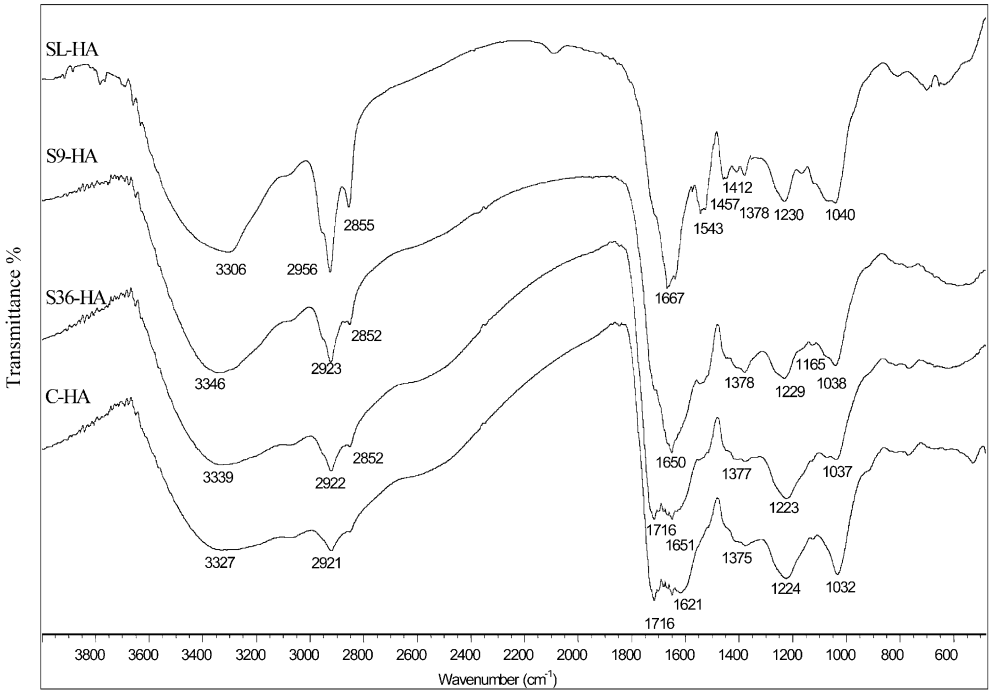

Fig. 1 Fourier transform infrared spectra of humic acids (HAs) isolated from sewage sludge (SL) (*SL-HA*), control soil (C) (*C-HA*), and SL-amended soils sampled 9 months (*S9-HA*) and

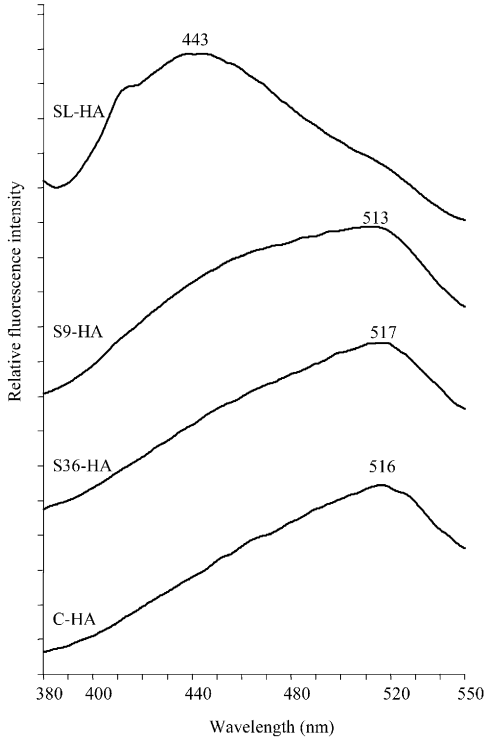

Fig. 2 Fluorescence emission spectra of SL-HA, C-HA, S9-HA and S36-HA . For abbreviations, see Fig. 1

intensity decreasing in the order: C-HA>S36-HA>S9-HA>SL-HA.

In general, results of the FTIR analysis agree with those obtained by elemental and functional group analyses suggesting a prevalent aliphatic character, a larger presence of N-containing groups and a smaller content of carboxyl and carbonyl groups in SL-HA with respect to soil HAs. Further, the trends of relative intensities of FTIR absorptions confirm those of compositional data in that the spectrum of S9-HA is more similar to that of SL-HA than to that of C-HA, whereas the opposite is true for the spectrum of S36-HA.

Fluorescence spectra

In agreement with previous results (Senesi et al. 1991), the RFI value of SL-HA is much larger than that of C-HA and S36-HA, but, surprising, slightly smaller than that of S9-HA (Table 3). The emission spectra (Fig. 2) show a unique typical broad band with the maximum centred at a wavelength that is much shorter (443 nm) for SL-HA than for any soil HA (513–517 nm). The sample S9-HA features the emission maximum at a slightly shorter wavelength than that of C-HA and S36-HA, and a broad shoulder that extends to shorter wavelengths.

The excitation and synchronous-scan spectra of SL-HA are very different from the corresponding spectra of soil HAs, and are characterized by a prominent peak in the intermediate wavelength region at about 390 nm and a series of less intense peaks and shoulders at short and long wavelengths (Figs. 3, 4, respectively). The excitation spectra of soil HAs exhibit a similar shape featuring two prominent peaks at long wavelengths (452 and 466 nm) and a shoulder at intermediate wavelength (about 395 nm) which show a slightly different relative intensity in the three samples (Fig. 3). Also the synchronous-scan spectra of soil HAs are similar showing a unique peak at long wavelength (at about 475 nm) associated with a broad

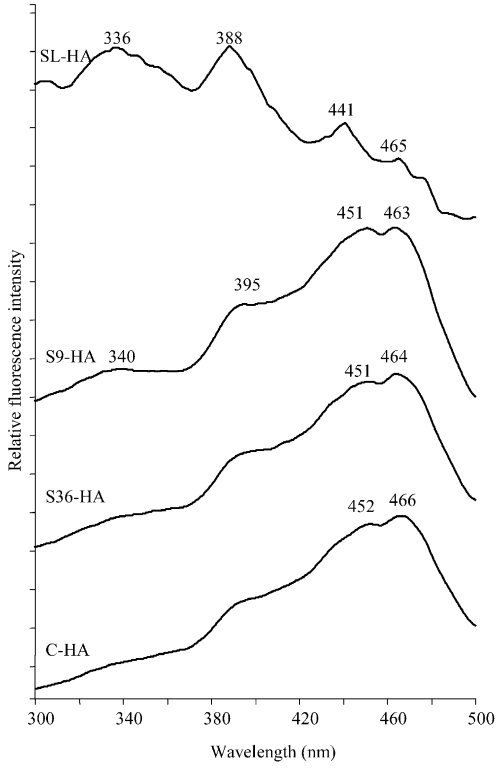

Fig. 3 Fluorescence excitation spectra of SL-HA, C-HA, S9-HA and S36-HA . For abbreviations, see Fig. 1

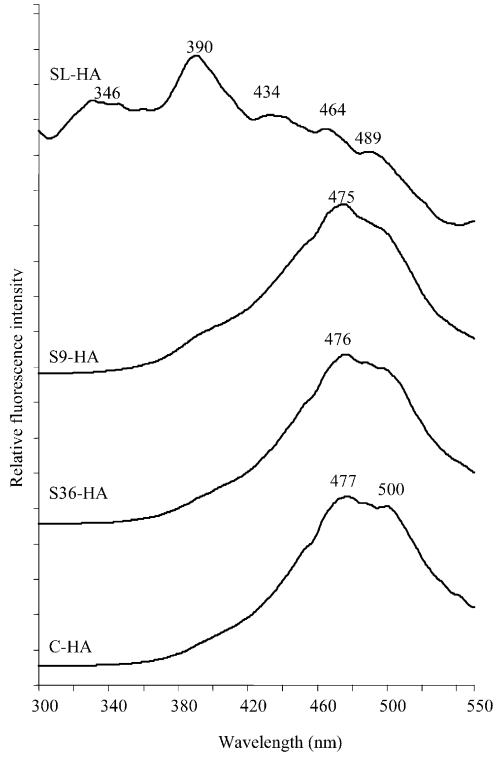

Fig. 4 Fluorescence synchronous-scan spectra of SL-HA, C-HA, S9-HA and S36-HA. For abbreviations, see Fig. 1

shoulder at longer wavelengths which extends up to 500 nm and whose relative intensity decreases in the order: C-HA, S36-HA, S9-HA (Fig. 4).

The large RFI value and short wavelength of the main fluorescence peaks of SL-HA suggest the presence of simple structural components of wide molecular heterogeneity, and aromatic polycondensation and humification to a small extent(Senesi et al. 1991). In contrast, the small RFI value, with the exception of sample S9-HA, and long wavelength of major peaks of soil HAs may be ascribed to the presence of an extended, linearly-condensed aromatic ring network and other unsaturated bond systems capable of a great degree of conjugation in extensively humified macromolecules (Senesi et al. 1991). The small differences in fluorescence properties exhibited by the three soil HAs show trends that confirm the compositional and infrared analyses results discussed above, indicating that the effects of SL amendment are more evident in sample S9-HA than S36-HA.

Soil basal respiration, microbial biomass C and qCO_2

Basal respiration is considered a good indicator of microbial activity in soil and a sensitive parameter for monitoring SOM mineralization (Anderson 1982; García and Hernández 1996). Soils in semiarid areas typically feature low microbial activity and SOM content (García et al. 1994a). The basal respiration value of sample S9 is much larger than those of the control samples and S36, and the value of sample S36 is slightly, but not significantly, larger than that of the control sample (Table 4). These results can presumably be ascribed to the labile C sources provided with the SL, which are used as easily decomposable substrates by soil microorganisms (Carpenter-Boggs et al. 2000). However, the added C sources are expected to be almost exhausted 36 months after SL application, as shown by the basal respiration value of sample S36, which is much smaller than that of S9 and similar to that of the control soil.

Similarly, the largest content of microbial biomass C was measured in sample S9, although there are no significant differences between any of the samples (Table 4). This result suggests that microbial biomass is not affected, especially in the long-term, by SL amendment.

In agreement with the results discussed above, the values of the qCO_2 are larger in sample S9 than in samples S36 and C (Table 4), which may be ascribed to the response of indigenous soil microbial biomass to the fresh input of organic C. The enhancement of qCO_2 may also, or alternatively, be attributed to the microbial stress caused by the larger amount of energy spent by microorganisms for their maintenance, which limits the incorporation of substrate C into their cell constituents (Leita et al. 1999). Further, the qCO_2 can be affected by changes in the composition of soil microflora due to heavy metal pollution (Brookes and McGrath 1984; Dahlin et al. 1997; Nannipieri et al. 1997; Moreno et al. 1999; Landi et al. 2000). As the ecosystem develops with time from the SL

Table 4 Basal respiration (*BR*), microbial biomass C (C_{bio}), metabolic quotient (qCO_2) and enzyme activities in the control soil and S9 and S36 . Values followed by the *same letter* within a column are not significantly different at $P \leq 0.05$. *DH* Dehydrogenase, *β-Glu* β-glucosidase, *INTF* iodo-nitrophenyl formazan. For other abbreviations, see Table 1

Soils	B.R. (μg C-CO_2 g^{-1} soil day^{-1})	C_{bio} (mg kg^{-1} soil)	qCO_2 (ng C-CO_2 g^{-1} h^{-1} μg^{-1} C_{bio})	Oxidoreductases		Hydrolases			
				DH (μg INTF g^{-1})	Catalase (μmol O_2 g^{-1} min^{-1})	Phosphatase (μmol p-nitro phenol g^{-1} h^{-1})	β-Glu (μmol p-nitrophenol g^{-1} h^{-1})	Urease (μmol NH_4^+ g^{-1} h^{-1})	Protease (μmol NH_4^+ g^{-1} h^{-1})
Control	14.6a	174a	3.4a	39.7a	17.1a	28a	79a	0.04a	0.13a
S9	49.5b	229a	9.1b	42.2a	20.5b	91b	145b	0.37b	0.45b
S36	17.8a	179a	4.2a	42.5a	16.5a	33a	86a	0.09a	0.21b

application, the qCO_2 declines due to the reduction of metabolic activity and the tendency to reach an energetic equilibrium in the system (Insam and Domsch1988).

Soil enzyme activities

Oxidoreductases are involved in oxidative processes in soils, and their activity mainly depends on the metabolic state of soil biota, thus they are considered as good indicators of soil microbial activity in semiarid areas (Garcia et al. 1994b). Of the two oxidoreductases measured, only catalase activity is significantly larger in soil S9, where the microflora is metabolically more active, whereas the dehydrogenase activity does not show any significant difference among the three soils (Table 4). Further, the residual effect of catalase activity in soil S36 is not significantly different from that of the control soil.

A significant increase in soil phosphatase and β-glucosidase activities, which play essential roles in the mineralization of organic P and C, respectively, was also measured in soil S9, with respect to the control soil and S36 (Table 4). This effect may be ascribed to the P and C substrates added to soil with the SL, which are able to increase the synthesis of these enzymes. Further, the possible presence in the SL of enzymes capable of promoting enzymatic activity in amended soil cannot be excluded. However, soil S36 does not show a significant increase in these enzyme activities compared to the control soil.

Both urease and protease activities are involved in the hydrolysis of N compounds to NH_4^+ using urea-type and low molecular weight protein substrates, respectively. These enzymes exhibit the greatest activity in soil S9, whereas in soil S36 only protease activity is significantly greater than that in the control soil (Table 4). The persistently large protease activity in soil S36 may be ascribed to the residual source of N substrates added with SL and/or to enhanced root exudation that can stimulate microbial activity and, in turn, intracellular enzyme activity. Root exudates can be a source of easily degradable N-compounds, e.g. amino acids and small peptides, able to induce protease synthesis (Lynch and Whipps 1990; Bolton et al. 1993; García-Gil et al. 2000). Further, protease activity can be possibly protected by the bonding of protease to soil colloids (Burns et al. 1982; García at al. 1994b; Nannipieri 1994).

Addition of SL appears, therefore, to promote microbiological and biochemical activities in soil, with enhancement of the rate of nutrient cycling by increasing SOM mineralization in amended soils. These results confirm that hydrolysis of organic compounds, such as proteinaceous, e.g. amino acids and peptides, and aliphatic, e.g. polysaccharides and fatty acids, materials added with SL to soil, contributes to the release of energy by microbial activity (Eivazi and Zakaria 1993). Further, the mineralization of biodegradable fractions of SL humic-like substances in soil may provide a source of energy for soil biota metabolism.

In conclusion, addition of SL to a degraded Mediterranean agroecosystem results in a number of short-term modifications to the compositional, structural, functional and reactive properties of the amended-soil HA and soil microbiological activities. With respect to the unamended soil HA, the HA isolated from the amended soil 9 months after SL application features a prevalent aliphatic character, a smaller oxygenated functional group content and larger contents of S- and N-containing components. The SL application induces an increase in soil microbial biomass content and its metabolic activity, which in turn accelerates the decomposition of the relatively fresh organic matter added with the SL. The significant increase in most enzymatic oxidative activities causes intense mineralization processes that possibly affect the chemical and physico-chemical properties of SL-HA added to soil leading after 36 months to a soil HA fraction with properties similar to those of the native soil HA fraction. This effect is confirmed by the microbial biomass content and activity also reaching values typical of the unamended soil 36 months after SL application.

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