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## Fluorescence analysis of humic and fulvic acids from two Brazilian oxisols as affected by biosolid amendment

Received: 22 July 2004 / Revised: 13 December 2004 / Accepted: 23 December 2004 / Published online: 3 March 2005  
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**Abstract** Conventional monodimensional fluorescence spectroscopy in the emission, excitation, and synchronous-scan modes and total luminescence spectroscopy have proven to be sensitive techniques for characterization and differentiation of humic acid (HA) and fulvic acid (FA) fractions isolated from an aerobically and anaerobically digested and limed biosolid, two layers of a sandy and a clayey Brazilian oxisol, and the corresponding biosolid-amended soils. The spectral patterns and the relative fluorescence intensities suggest greater molecular heterogeneity, less aromatic polycondensation, and less humification of biosolid HA and FA compared with soil HA and FA. However, the differences are smaller for the FA fractions than for the HA fractions. Fluorescence properties of soil HA and FA differ slightly as a function of soil type and soil layer. Biosolid application causes a shift to shorter wavelengths of the main fluorescence peaks and marked variation of the relative fluorescence intensities of HA and FA isolated from amended soils. These results suggest that molecular components of relatively small molecular size, with a low level of aromatic polycondensation, and low degree of humification present in biosolid HA and FA are partially and variously incorporated into amended soil HA and FA. In general, these modifications seem to be smaller in HA and FA from the clayey soil layers than in those from the sandy soil layers, possibly because of protective effects exerted by clay minerals of native soil HA and FA against disturbances caused by biosolid application.

**Keywords** Biosolid · Oxisols · Humic acids · Fulvic acids · Monodimensional fluorescence spectroscopy · Total luminescence spectroscopy

### Introduction

Humic substances are the most widespread and ubiquitous natural nonliving organic materials in all terrestrial and aquatic environments, and represent the major fraction of soil organic matter [1]. They are a physically and chemically heterogeneous mixture of biogenic organic polymers of mixed aliphatic and aromatic nature, rich in carboxylic and phenolic functional groups, formed by secondary synthetic reactions (the humification process) of biomolecules originating from the decay process, transformations of dead organisms, and microbial activity [2]. The major components of humic substances are humic acids (HA), the portion soluble in alkali and insoluble in acid, and fulvic acids (FA), the portion soluble in alkali and in acid [1]. These materials are known to contribute substantially to maintaining global soil fertility, and thus agricultural production, and to protecting the soil from degradation and contamination [1].

Soils poor in organic matter, for example tropical soils, including oxisols, may benefit of amendment with organic waste materials of different nature and source, including municipal sewage sludges, also called “biosolids”. Biosolid recycling as soil organic amendment is also a convenient alternative to landfill disposal, especially in densely populated areas such as the metropolitan São Paulo city in Brazil, where daily biosolid production is extremely large and disposal sites are less and less available. However, control of the chemical quality of organic matter, especially the HA-like and FA-like fractions contained in the biosolid, is of crucial importance to ensure the environmentally safe and agronomically efficient use of biosolid as a soil amendment. In practice, the compositional, structural and

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functional properties of the HA-like and FA-like fractions in the biosolid should resemble, as much as possible, those of native soil HA and FA [3].

Conventional monodimensional fluorescence spectroscopy in the emission, excitation, and synchronous-scan modes and three-dimensional fluorescence or total luminescence (TL) spectroscopy are proven powerful techniques for characterization and differentiation of HA and FA as a function of their nature and origin [4, 5]. Although fluorescent structures constitute minor components of HA and FA macromolecules, fluorescence analysis can provide important information, on a comparative basis, about the structural and functional similarity and/or differences of these materials, which can also be related to their degree of polymerization and humification.

In previous work [6] HA fractions isolated from the same biosolid sample and nonamended and biosolid-amended oxisols used in this work were characterized to determine their elemental composition and structural and functional properties. The objective of this study was to extend the chemical characterization of the HA fractions to the FA fractions by using both monodimensional fluorescence and TL spectroscopy, to evaluate the impact of biosolid amendment on the HA and FA of two layers of two Brazilian oxisols.

## Materials and methods

### Biosolid and soils

The biosolid (B) used in this study was semisolid sludge obtained from sewage effluents subjected to conventional aerobic and anaerobic treatment and then limed with CaO and FeCl<sub>3</sub> in the Barueri sewage treatment plant located in a large industrial area in the metropolitan region of São Paulo State, Brazil.

The soils used were a sandy typic hapludox and a clayey typic hapludox [7], commonly named oxisols, cropped with corn, either non-amended (TH1 and TH2) or amended with an amount of biosolid equivalent to 390 Mg ha<sup>-1</sup> on a dry-weight basis (TH1B and TH2B). The soils were sampled 6 months after biosolid application from the surface (s, 0–25 cm) and subsurface (ss, 25–50 cm) layers, then dried at 60°C, crushed, and sieved at 2 mm. Some relevant physical and chemical properties of the two soils are shown in Table 1; a detailed discussion of these data is available elsewhere [6].

**Table 1** Some physical and chemical properties of the soils examined

Soil sample	Clay (g kg <sup>-1</sup> )	Silt (g kg <sup>-1</sup> )	Sand (g kg <sup>-1</sup> )	pH (H <sub>2</sub> O)	C <sub>org</sub> (g kg <sup>-1</sup> )	CEC (mmol <sub>(c)</sub> kg <sup>-1</sup> )
TH1s	151	31	818	5.8 ± 0.2	5.8	18.7 ± 0.5
TH1ss	161	7	832	5.7 ± 0.2	5.5	16.5 ± 0.3
TH2s	593	109	298	5.9 ± 0.1	12.9	41.2 ± 1.1
TH2ss	616	108	276	5.8 ± 0.1	13.6	40.6 ± 1.6

### Isolation of humic and fulvic acids

The HA and FA were isolated from the soil samples by a conventional procedure [8] described in detail in a previous paper [6]. In brief, each soil sample was extracted three successive times with a fresh 0.5 mol L<sup>-1</sup> NaOH and 0.1 mol L<sup>-1</sup> Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> solution under N<sub>2</sub> atmosphere using a soil to extractant ratio of 1:5. After centrifugation at 6000g for 20 min and filtration through a Whatman (glass microfibre) GF/C filter, the combined extracts were acidified to pH 1.0 with HCl, kept standing for 24 h, and centrifuged as above, to separate precipitated HA from supernatant containing dissolved FA. The HA precipitates were then purified by three successive redissolutions in 50 mL 0.5 mol L<sup>-1</sup> NaOH, centrifugation, and reprecipitation by acidification to pH 1.0. Finally, the HA were suspended in bidistilled water, dialysed using a Spectrapore membrane (size exclusion limit 6000–8000 Da), freeze-dried, and lyophilized. The FA fractions were analysed in the dissolved state without further purification.

A similar procedure was used to isolate the HA and FA fractions from the biosolid sample using a biosolid to extractant ratio of 1:20.

### Fluorescence spectra of humic and fulvic acids

Fluorescence spectra were recorded on aqueous solutions of 100 mg L<sup>-1</sup> HA after overnight equilibration at room temperature, and adjustment to pH 8.0 with 0.05 mol L<sup>-1</sup> NaOH, and directly on the dissolved FA fractions, using a Hitachi model F-4500 luminescence spectrophotometer. Monodimensional emission spectra were recorded over the range 380–550 nm at a constant excitation wavelength of 360 nm. Excitation spectra were recorded over the range 300–500 nm at a fixed emission wavelength of 520 nm. Synchronous-scan excitation spectra were measured by scanning simultaneously both the excitation and the emission wavelengths (from 300 to 550 nm), while maintaining a constant, optimized wavelength difference  $\Delta\lambda = \lambda_{em} - \lambda_{exc} = 18$  nm [4].

The TL spectra were obtained in the form of excitation/emission matrix (EEM) spectra (or contour maps) by scanning the wavelength emission over the range 400 to 600 nm, while the excitation wavelength was increased sequentially by 5-nm steps from 300 to 500 nm. The EEM spectra (or contour maps) were generated from TL spectral data by using Noesys 2.4 software.

The overall relative fluorescence intensity was expressed in arbitrary units and either calculated as the unitless reciprocal of the gain used to normalize each emission spectrum ( $\text{RFI}_{\text{em}}$ ) [4], or obtained experimentally from TL spectra ( $\text{RFI}_{\text{TL}}$ ).

## Results

### Monodimensional fluorescence spectra

The monodimensional fluorescence spectra in the emission, excitation, and synchronous-scan modes of the HA and FA examined are shown in Figs. 1 and 2, respectively, and the corresponding  $\text{RFI}_{\text{em}}$  values are listed in Table 2. All emission spectra feature a typical unique broad band with a flat maximum whose wavelength is positioned at 460 nm for the biosolid HA and at a much longer similar wavelength (from 542 to 549 nm) for all soil HA. The wavelength emission maxima of soil FA are typically shorter than those of the corresponding HA and are centred at about the same wavelength of that of biosolid FA (480 nm). The emission maxima of FA from amended soils have a net shift toward shorter wavelengths (466 nm) whereas no variation is observed for those of the HA counterparts.

The  $\text{RFI}_{\text{em}}$  values of biosolid HA and FA are much smaller than those of soil HA and FA, and the  $\text{RFI}_{\text{em}}$  values of HA and FA from sandy soils TH1 are smaller than those of the corresponding HA and FA from clayey soils TH2. As an effect of the biosolid addition, the  $\text{RFI}_{\text{em}}$  values of HA from amended soils are generally smaller than those of the corresponding HA from non-amended soils, whereas the opposite is true for  $\text{RFI}_{\text{em}}$  values of FA.

The excitation spectrum of the biosolid HA contains two main peaks at 358 and 438 nm, and a shoulder at about 460 nm, whereas that of biosolid FA contains a unique broad band with the maximum centred at 409 nm. In contrast, soil HA are characterized by a unique excitation peak centred at a similar wavelength ranging from 450 to 459 nm whereas soil FA give a main broad peak in the short-wavelength region (at about 360 nm for TH1-FA and at about 378 nm for TH2-FA) and a shoulder at longer wavelength (at about 440 nm). After biosolid addition, a slight shift toward shorter wavelengths is observed for the main excitation peak of HA and FA from sandy soils TH1, whereas no substantial change is observed in the excitation spectra of HA and FA from clayey soils TH2.

The synchronous-scan spectra of HA differ as a function of their source and from those of the corresponding FA. In particular, the synchronous-scan spectrum of the biosolid HA contains a dominant peak at 465 nm and three minor peaks at shorter (330 and 385 nm) and longer (508 nm) wavelengths, whereas the spectrum of the corresponding FA contains a broad peak with a maximum at 461 nm accompanied by two shoulders at shorter and longer wavelengths (390 and

525 nm, respectively). The HA from nonamended sandy soils TH1 give a unique broad peak centred at about 510 nm, which shifts to 485 nm with a shoulder at 509 nm in HA from the corresponding amended soils. The HA from nonamended clayey soils TH2 give a main peak at 486 or 491 nm (associated with a shoulder at about 508–509 nm), which also shifts to shorter wavelength (483 or 485 nm) in HA from the corresponding amended soils. The FA from all soils give a main peak at about 470 nm and one or two minor peaks or shoulders in the intermediate and short wavelength regions (at about 390 and 365 nm for TH1-FA and at about 400 nm for TH2-FA). Although the synchronous-scan peaks of FA from amended soils do not vary in their position compared with the corresponding FA from nonamended soils, the intensity of the peak at long wavelength generally decreases relative to those at intermediate and short wavelengths, which is more evident for TH1-FA.

### Total luminescence spectra of humic acids

The TL spectra in the form of EEM spectra (or contour maps) of the HA and FA examined are shown in Figs. 3 and 4, respectively, and the excitation/emission wavelength pairs (EEWP) of the main peak maxima and the corresponding  $\text{RFI}_{\text{TL}}$  are listed in Table 3. The EEM spectrum of biosolid HA contains a major peak at a short wavelength pair ( $355_{\text{ex}}/435_{\text{em}}$ ), and a less intense peak at a longer wavelength pair ( $440_{\text{ex}}/520_{\text{em}}$ ) whereas that of the corresponding FA contains a unique fluorophore at an intermediate EEWP value ( $395_{\text{ex}}/490_{\text{em}}$ ). Similar to  $\text{RFI}_{\text{em}}$ , the  $\text{RFI}_{\text{TL}}$  of biosolid HA is much smaller than that of the corresponding FA.

The EEM spectra of nonamended soil HA are characterized by a unique peak centred in the long wavelength region, that is, at  $465_{\text{ex}}/545_{\text{em}}$  and  $475_{\text{ex}}/555_{\text{em}}$ , respectively, for TH1s-HA and TH1ss-HA, and at  $465_{\text{ex}}/545_{\text{em}}$  for both TH2s-HA and TH2ss-HA. Differently, the EEM spectra of soil FA contain a major peak at short wavelengths ( $325_{\text{ex}}/435_{\text{em}}$  for TH1-FA and  $360_{\text{ex}}/470_{\text{em}}$  for TH2-FA), and a less intense peak at longer wavelengths (at  $435_{\text{ex}}/525_{\text{em}}$  and  $445_{\text{ex}}/525_{\text{em}}$  for TH1-FA and  $445_{\text{ex}}/510_{\text{em}}$  and  $435_{\text{ex}}/525_{\text{em}}$  for TH2-FA). Similar to  $\text{RFI}_{\text{em}}$ , the  $\text{RFI}_{\text{TL}}$  values of biosolid HA and FA are much smaller than those of soil HA and FA, respectively. Further, the  $\text{RFI}_{\text{TL}}$  values of TH1-HA and TH1-FA are smaller than those of the corresponding TH2-HA and TH2-FA, respectively.

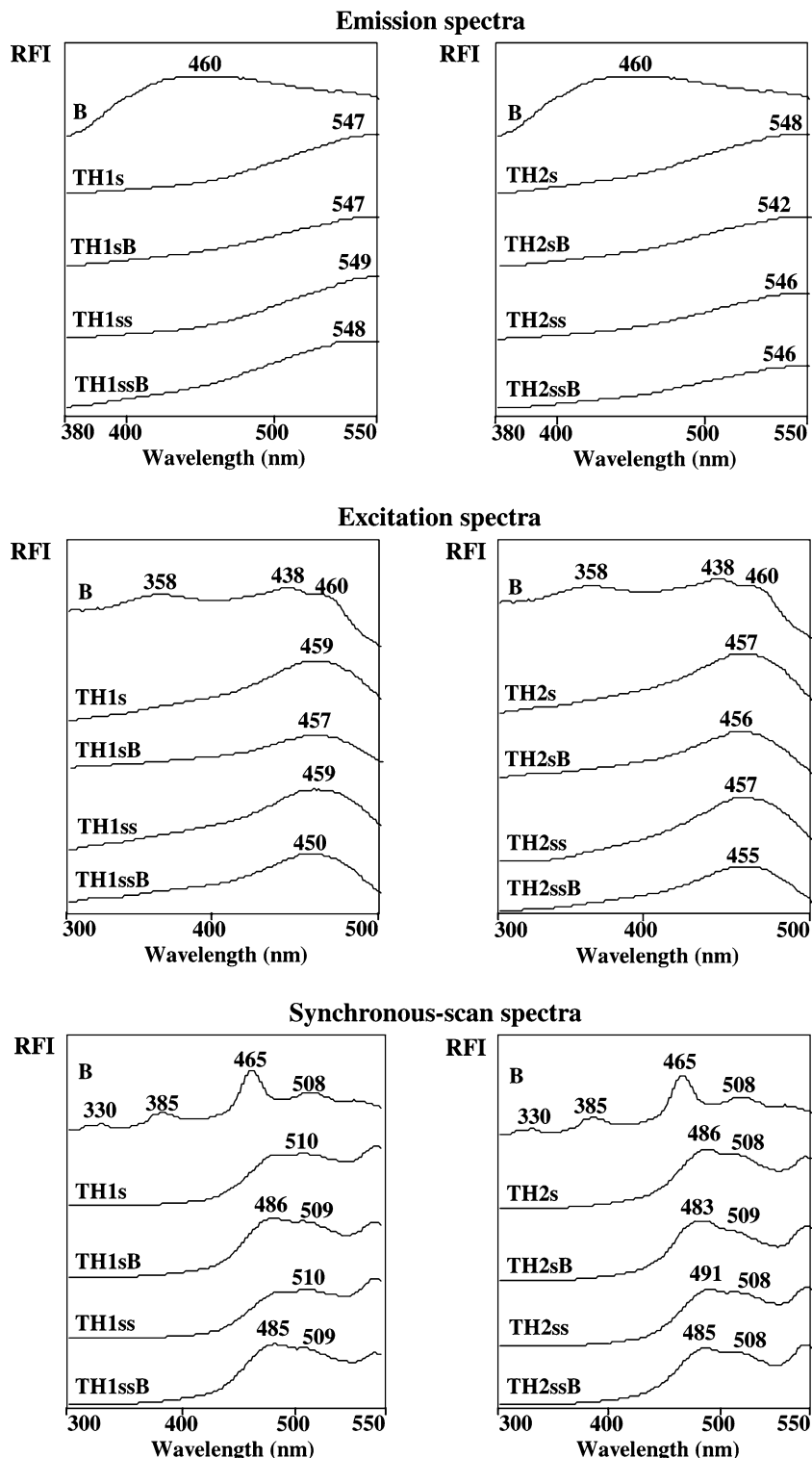
The EEM spectra of amended soils HA and FA have important differences from those of the corresponding nonamended soils HA and FA. In particular, for HA from amended soils EEWP generally shift toward shorter wavelengths and there is a marked reduction of  $\text{RFI}_{\text{TL}}$  values, whereas the corresponding FA spectra feature a slightly-shifted main peak at short EEWP, the disappearance of the peak at long EEWP, and an increase of  $\text{RFI}_{\text{TL}}$  values.

## Discussion and conclusions

Soil HA are characterized by typical long wavelengths of emission maxima, relatively more intense excitation, synchronous-scan and TL peaks at long rather than short and intermediate wavelengths, and small  $RFI_{em}$

values. These results confirm the presence in soil HA of large molecular size components rich in extended, linearly condensed aromatic ring networks and other unsaturated bond systems capable of a great degree of conjugation, and bearing electron-withdrawing substituents such as carbonyl and carboxyl groups [4, 9]. On the other hand, soil FA feature emission maxima at

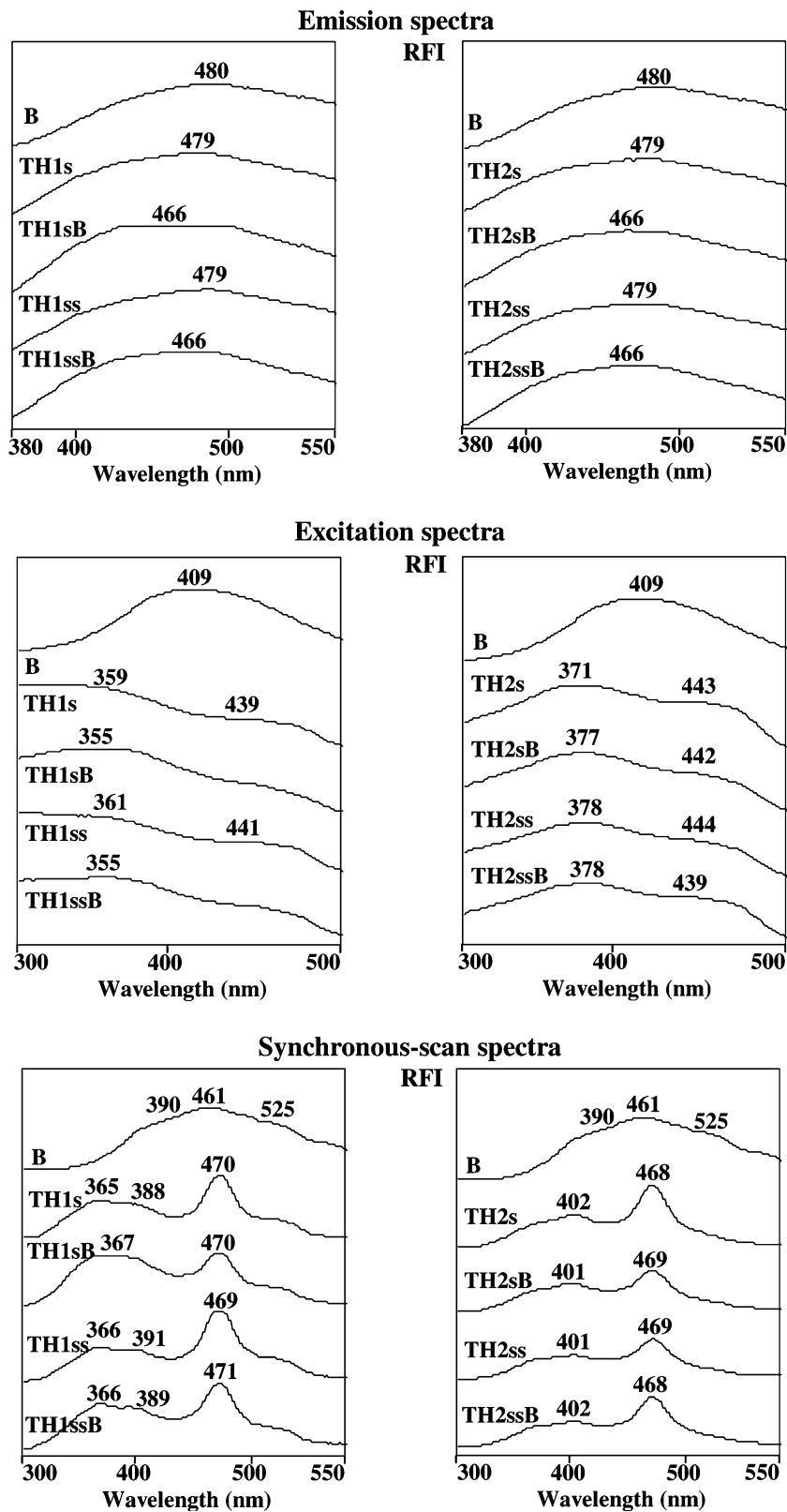
**Fig. 1** Monodimensional fluorescence spectra of humic acids isolated from the biosolid (B), the nonamended surface (TH1s, TH2s) and subsurface (TH1ss, TH2ss) soils, and the corresponding biosolid-amended surface (TH1sB, TH2sB) and subsurface (TH1ssB, TH2ssB) soils



short wavelengths, the prevalence of excitation, synchronous-scan and TL peaks at short wavelengths, and large  $\text{RFI}_{\text{em}}$  values. These results may be ascribed to the presence of simple structural components of small

molecular size bearing electron-donating substituents such as hydroxyl, methoxy, and amino groups, and to small levels of aromatic polycondensation and conjugated chromophores [4, 9].

**Fig. 2** Monodimensional fluorescence spectra of fulvic acids isolated from the biosolid (*B*), the nonamended surface (*TH1s*, *TH2s*) and subsurface (*TH1ss*, *TH2ss*) soils, and the corresponding biosolid-amended surface (*TH1sB*, *TH2sB*) and subsurface (*TH1ssB*, *TH2ssB*) soils



**Table 2** Relative fluorescence intensity values ( $RFI_{em}$ , arbitrary units) calculated from the emission spectra of the humic acids and fulvic acids examined

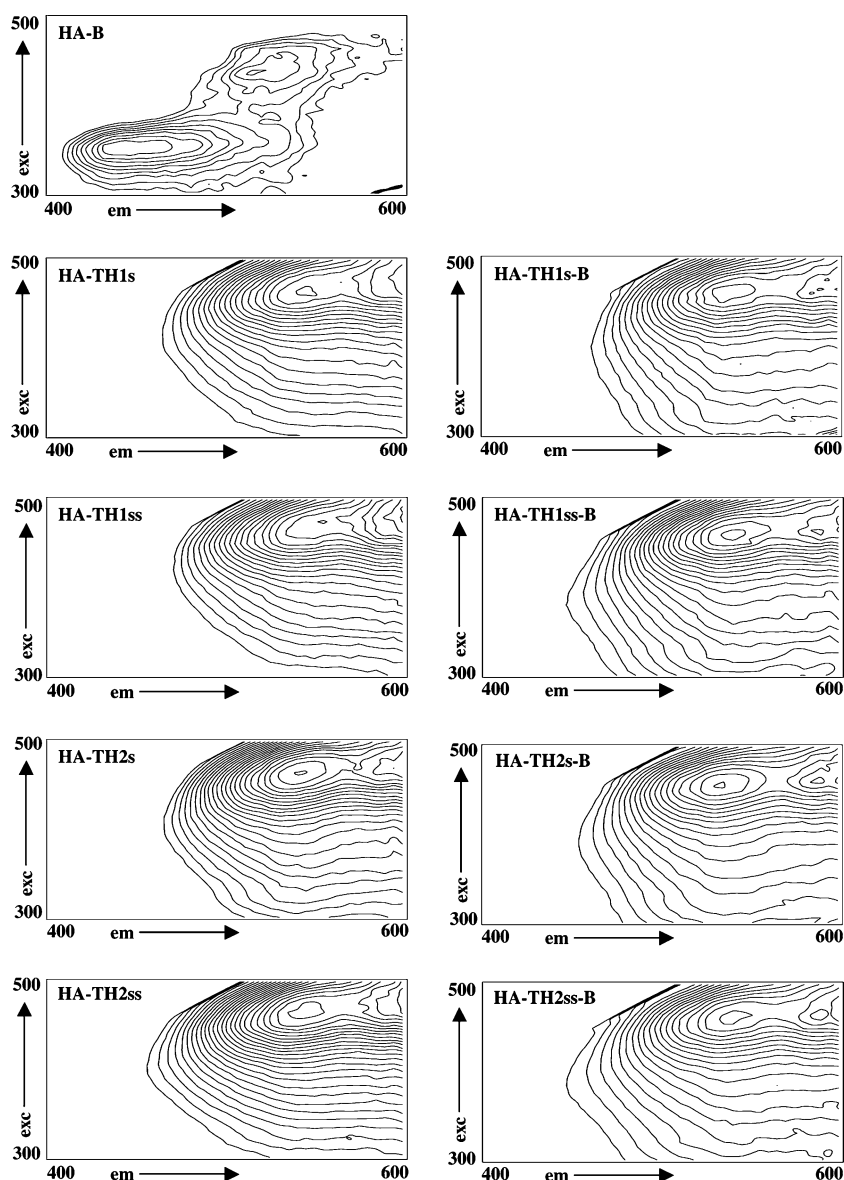
Origin of samples	Humic acids	Fulvic acids
Biosolid	158	186
TH1s	284	384
TH1sB	239	450
TH1ss	293	369
TH1ssB	335	423
TH2s	411	824
TH2sB	333	735
TH2ss	310	679
TH2ssB	287	691

The fluorescence properties of HA and FA differ slightly as a function of soil type and soil layer. In particular, the smaller  $RFI_{em}$  and  $RFI_{TL}$  values of TH1-HA and TH1-FA, compared with those of the corresponding

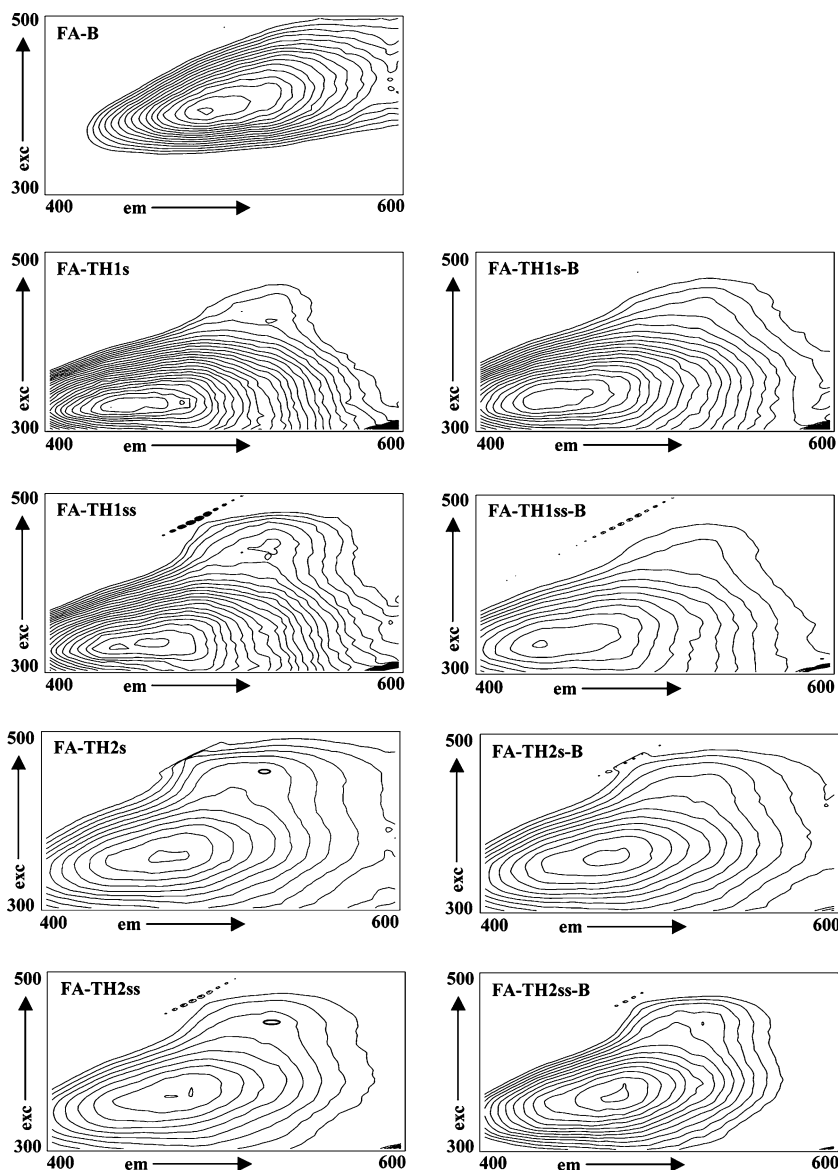
TH2-HA and TH2-FA, suggest the presence in the former samples of more polycondensed aromatic systems of larger molecular size and richer in conjugated chromophores. Biosolid HA and FA also differ in their fluorescence properties, although the differences are not simple to interpret, because these materials are relatively fresh, not-well differentiated molecularly, and low-humified compared with soil HA and FA, as discussed below.

The fluorescence properties of biosolid HA and FA differ markedly from those of soil HA and FA, which implies different structural and functional chemical properties. In particular, the biosolid HA features an emission maximum at much shorter wavelength and more complex excitation, synchronous-scan and TL spectra compared with soil HA. Further, biosolid HA and FA have much smaller  $RFI_{em}$  and  $RFI_{TL}$  values than soil HA and FA. These results suggest greater molecular heterogeneity, a lower level of conjugated chromophores and aromatic polycondensation, and a

**Fig. 3** Total luminescence spectra of humic acids isolated from the biosolid (B), the nonamended surface (TH1s and TH2s) and subsurface (TH1ss and TH2ss) soils, and the corresponding biosolid-amended surface (TH1sB and TH2sB) and subsurface (TH1ssB and TH2ssB) soils



**Fig. 4** Total luminescence spectra of fulvic acids isolated from the biosolid (*B*), the nonamended surface (*TH1s* and *TH2s*) and subsurface (*TH1ss* and *TH2ss*) soils, and the corresponding biosolid-amended surface (*TH1sB* and *TH2sB*) and subsurface (*TH1ssB* and *TH2ssB*) soils



**Table 3** Excitation/emission wavelength pairs (EEWP, nm/nm) of the main peak maxima in total luminescence spectra and the corresponding relative fluorescence intensities (RFI<sub>TL</sub>, arbitrary units) of the humic acids and fulvic acids examined

Origin of sample	Humic acids		Fulvic acids			
	EEWP	RFI <sub>TL</sub>	EEWP	RFI <sub>TL</sub>	EEWP	RFI <sub>TL</sub>
Biosolid	355/435 <sup>a</sup>	156 <sup>a</sup>	395/490	315	n.d. <sup>b</sup>	n.d.
TH1s	465/545	697	325/435	462	435/525	170
TH1sB	460/545	475	330/440	498	n.d.	n.d.
TH1ss	475/555	723	325/435	401	445/525	194
TH1ssB	460/540	652	330/435	454	n.d.	n.d.
TH2s	465/545	872	360/470	792	445/510	477
TH2sB	455/535	663	355/465	716	n.d.	n.d.
TH2ss	465/545	853	360/470	652	435/525	394
TH2ssB	465/545	644	355/470	664	n.d.	n.d.

<sup>a</sup>Major peak; the minor peak features EEWP=440<sub>exc</sub>/520<sub>em</sub> and RFI = 132

<sup>b</sup>Not detected

smaller degree of humification of biosolid HA and FA compared with soil HA and FA. However, the similar wavelengths of the emission maxima and of the main synchronous-scan peaks of biosolid FA and soil FA suggest the existence of less structural and functional molecular differences between these samples than between the corresponding HA.

Comparison of fluorescence properties of HA and FA from biosolid-amended soils with those of the corresponding HA and FA from nonamended soils indicates that both materials are affected to a different extent by biosolid addition, as a function of the nature of the material and the type of soil. In general:

- a shift to shorter wavelengths is generally observed for the emission maximum of FA, the main excitation peak of TH1-HA and TH1-FA, and the main synchronous-scan and TL peaks of the HA;

- the intensity of the synchronous-scan peak at long wavelength of FA, and especially of TH1-FA, decreases relatively to that of peaks at intermediate and short wavelengths; and
- the  $RFI_{em}$  and  $RFI_{TL}$  values of HA decrease, whereas those of FA increase.

These results suggest that molecular components of relatively small molecular size, small level of aromatic polycondensation, and low degree of humification present in biosolid HA and FA are partially and differently incorporated into amended-soil HA and FA, thus modifying their structural and functional properties. In particular, the HA fractions would be enriched with electron-withdrawing substituents, for example carboxyl and carbonyl groups, whereas the FA fractions would preferentially incorporate electron-donating substituents such as hydroxyl, methoxy, and amino groups.

In general, the modifications caused by biosolid amendment seem to be greater in the HA and FA of sandy soils TH1 than in those of clayey soils TH2. These results may be related to the protective effect of clay minerals in clayey soils on native soil HA and FA against external factors of disturbance such as biosolid application. On the other hand, native soil HA and FA would be more affected by the biosolid addition in the sandy soil poor in clay minerals.

In conclusion, both conventional monodimensional fluorescence spectroscopy and total luminescence spectroscopy are proven to be sensitive techniques for characterization and differentiation of the HA and FA fractions of soils and biosolids, and for evaluation of the

impact of biosolid HA and FA on native soil HA and FA.

**Acknowledgements** This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), SP, Brazil, and the Dipartimento di Biologia e Chimica Agroforestale ed Ambientale, Università di Bari, Italy.

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