Talanta 134 (2015) 453–459

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

The use of solid-phase fluorescence spectroscopy in the characterisation of organic matter transformations

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ARTICLE INFO

Article history:
Received 14 July 2014
Received in revised form
19 November 2014
Accepted 22 November 2014
Available online 3 December 2014

Keywords:
Solid-phase fluorescence spectroscopy
Organic matter
Compost
13C CPMAS NMR
Rock-Eval pyrolysis

ABSTRACT

Given its high sensitivity and non-destructive nature, fluorescence excitation–emission matrix (EEM) spectroscopy is widely used to differentiate changes and transformations of dissolved or water-extracted organic matter (OM) in natural environments. The same technique applied directly on solid samples (solid-phase fluorescence spectroscopy, SPF-EEM) provides accurate results when used with pharmaceutical products or food samples, but only a few studies have considered natural OM. This study reports on the use of SPF-EEM on solid compost samples and emphasises the way the different maturation phases can be distinguished with fluorophores closely resembling those found in dissolved samples. A very good correlation has been found with data from Rock-Eval pyrolysis, nuclear magnetic resonance (^{13}C CPMAS NMR), and humic-fulvic acid ratios determined by conventional NaOH-extraction. SPF-EEM appears as a much simpler method than the conventional ones to detect transformations in natural OM samples with low mineral contents. However, direct application to soil samples requires some additional studies.

1. Introduction

Human activities generate large amounts of solid wastes, the handling of which being a problem of increasing concern worldwide. According to Moran Viezra et al. [1], the biodegradable fraction in solid wastes is processed more and more through composting, which accelerates, in controlled form, the natural processes of biological transformation of organic matter (OM). Composting of organic wastes is a bio-oxidative process involving the mineralisation and partial humification of OM, leading to a stabilised final product, free of phytotoxicity and pathogens and with some humic properties [2]. However, the application of immature compost can result in inhibited seed germination, root destruction, suppressed plant growth, and a decrease in oxygen concentration and redox potential [3]. Therefore, assessment of compost maturity is of utmost importance for achieving high quality compost to guarantee its marketability [4]. Thus, the development of new and/or simple methodologies to monitor the evolution and maturity of compost is clearly an issue of high relevance.

Several criteria and methods have been proposed to assess compost maturity, including either simple empirical observations (odour reduction, mixture darkening, etc.) or rigorous analytical methods (biological, chemical and physicochemical, etc.) including pH, electrical conductivity, total organic C and N, cation exchange capacity (CEC), humified OM content, enzymatic activities, O_2 consumption, etcetera. [5–7].

In a previous study, numerous chemical and biological parameters including moisture, pH, Corg, Norg, C/N, OM, humic-like acid (HA), fulvic-like acid (FA), respiration, cellulase, protease, and phenoloxidase activities were monitored during a complete composting process lasting six months [6]. Results revealed the existence of two development phases within the composting processes. The initial phase (4 to 50–60 days) was characterised by an intensive degradation and a rapid increase in temperature. Both C/N ratio and OM content decreased sharply. The second phase (up to 146 days) was characterised by the stabilisation of C/N ratio and OM content, a decrease in all biological activities and an increase in the humification process occurring within the OM, with a notably high increase of the HA/FA ratio, which tripled from 0.54 to 1.61 between 4 and 146 days. This decline in biological activity was explained by a quantitative and qualitative reduction of nutrient sources, which became a limiting factor. Indeed, as shown by ^{13}C CPMAS NMR on the same samples [7], peaks at 40 and 35 ppm,

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http://dx.doi.org/10.1016/j.talanta.2014.11.056
0039-9140/

assigned to the CH_2 groups of proteins and lipids, which are easily degradable compounds, decrease rapidly during composting. Similarly, an increase in aromaticity (aromatic C+phenolic C) by ^{13}C NMR emphasises a clear preference for easily biodegradable C compounds by microorganisms.

However, one single parameter cannot be taken as an index of compost maturity and several methods are often employed. This approaches is time-consuming or expensive when a large number of samples are involved [6]. Moreover, many of these methods require complex sample preparation and/or specific reactants. In contrast, spectroscopic techniques present some advantages for example they are rapid (can be used to monitor process dynamics), non-destructive (facilitate measurements on intact sample structures), environmentally friendly (no use of chemicals and no harm to the environment) and allow the measurement of several quality parameters simultaneously. In the last ten years, many different spectroscopic methods have been investigated to characterise OM, for example, mid and near-infrared, nuclear magnetic resonance (NMR), UV–visible and fluorescence spectroscopy [8–11]. However, for routine applications, some of these techniques suffer from major disadvantages. NMR requires advanced scientific skills and expensive analytical equipment. UV-visible spectroscopy is usually not informative, sensitive or selective enough and moreover, preliminary extractions are needed. Although mid and near-infrared spectroscopy can be applied directly on solid samples, fluorescence spectroscopy gives a different type of information (fluorophores vs. vibrational-active functional groups) [9].

Fluorescence Excitation–Emission Matrix (EEM) spectroscopy is among the most promising tools for characterising heterogeneous OM by providing comprehensive information on the composition, properties, and behaviour of the samples [10]. Given its high sensitivity, selectivity, and non-destructive nature, EEM is widely used to differentiate the changes and transformations of OM in natural environments [11]. However, EEM is mostly applied on water-extractable organic matter (WEOM). WEOM is interesting for the aquatic environment and dissolved OM in marine and freshwaters, as well as in raw and treated wastewater. However, because of this extraction step, which produces some bias, WEOM is clearly less interesting for soil OM or organic wastes such as compost. From this postulate, we choose to apply fluorescence spectroscopy directly on solid samples. Solid-phase fluorescence spectroscopy (SPF-EEM) has been shown to be able to provide accurate results in solid samples such as pharmaceutical products [12], crushed nuts and sesame seeds [13], and chicken meat [14]. But very few studies can be found on natural OM [9,15,16].

Nevertheless, there is still a question pending: can the major fluorophores found by SPF-EEM be identified? To answer this question, ^{13}C Nuclear Magnetic Resonance Spectroscopy in the solid state (^{13}C CPMAS NMR) and RockEval pyrolysis (RE pyrolysis) have been used as reference tools. The aim is to link SPF fluorophores to organic matter components identified by these reference tools.

The aim of this study is to evaluate the applicability of SPF-EEM to OM of compost (green waste and sewage sludge). OM from compost samples has been used for the following reasons: 1) contrary to soils, compost variability only comes from transformation of OM and variability of inherited parent materials; 2) composting processes are well described and referenced in the literature; 3) the samples used have already been characterised in previous works [6,7] and are well constrained.

2. Materials and methods

2.1. Experimental materials

Composts were obtained from local dewatered digested municipal sewage sludge, green wastes, and pine barks at a 1:1:1 v/v ratio (Company Biotechna, Ensuès, Bouches du Rhône, France). Pine barks were incorporated into other biowastes to improve aeration during the process. The mixture was composted for 20 days in impervious boxes (100 m^3) with forced aeration, and then stored in windrows (10 m long, 4 m high, and 5 m deep) on a composting platform for six months. The heaps were mixed several times during the process to promote OM humification. Approximately 1 kg of homogenised compost was collected from each windrow at eight different stages of composting (4, 18, 40, 67, 84, 101, 114, and 128 days) with four replicates for 32 samples in total. All samples were sieved (2 mm mesh). Samples were freeze-dried and ground with a Cyclotec 1093 mill (FOSS) to 1 mm size.

2.2. Fluorescence spectroscopy

Fluorescence EEM was recorded using a spectrofluorimeter (FluoroLog-3, Horiba) equipped with a front surface accessory specifically designed for solid samples. The incidence angle of the excitation radiation was set at 30° to ensure that reflected light, scattered radiation, and depolarisation phenomena were minimised. A 450 W xenon lamp CW emitted a pulsed radiation from 250 to 500 nm and the emission wavelengths ranged between 300 and 600 nm. The step size for excitation was 5 nm and for the emission 2.5 nm. Slit widths of the excitation and emission monochromator were set at 5 nm with a 0.1 s integration time. Emission monochromator scan speed was 150 $nm.s^{-1}$. EEMs were acquired in triplicates on different sides of the cuvettes. If necessary, spectra were corrected for inner filtering using dilution of sample in sodium carbonate, which does not emit any fluorescence when excited from 200 to 600 nm. All samples were blank-corrected and the two bands corresponding to the first- and the second-order Rayleigh scattering were detected and eliminated using a simple algorithm.

2.3. Statistical analysis

The interpretation of fluorescence spectral data is complex due to the presence of many fluorophores in the same EEMs. PARAFAC was applied to SPF-EEM to facilitate their interpretation. PARAFAC is a statistical tool used to decompose a complex mixture of fluorophores into non-covarying components, without any assumptions about their spectral shape or component number. From PARAFAC modelling, one may obtain excitation and emission spectra of components controlling EEMs of fluorescence and cores of fluorescent components proportional to their concentrations. The variation of concentration scores provides a quantitative basis for monitoring changes in concentrations of fluorescent components obtained within the PARAFAC model [17]. PARAFAC analyses were carried out with the Progmeef program provided by R. Redon and S. Mounier (PROTEE laboratory, Univ. Toulon, France) based on MatlabTM software (Matlab 2012b). The model was run with non-negativity constraints applied to each dimension, considering that negative values of these parameters have no physical meaning.

The relationships between concentration scores from PARAFAC analysis and major classes of organic constituents given by RE pyrolysis and ^{13}C CPMAS NMR were calculated using Pearson coefficients of determination (r) using R software. Significant coefficients were retained for $p\text{-}value < 0.05$.

3. Results & discussions

3.1. EEM – PARAFAC components of compost samples

The three dimensional EEM fluorescence spectra of bulk compost samples between 4 and 128 days are shown in Fig. 1. All of

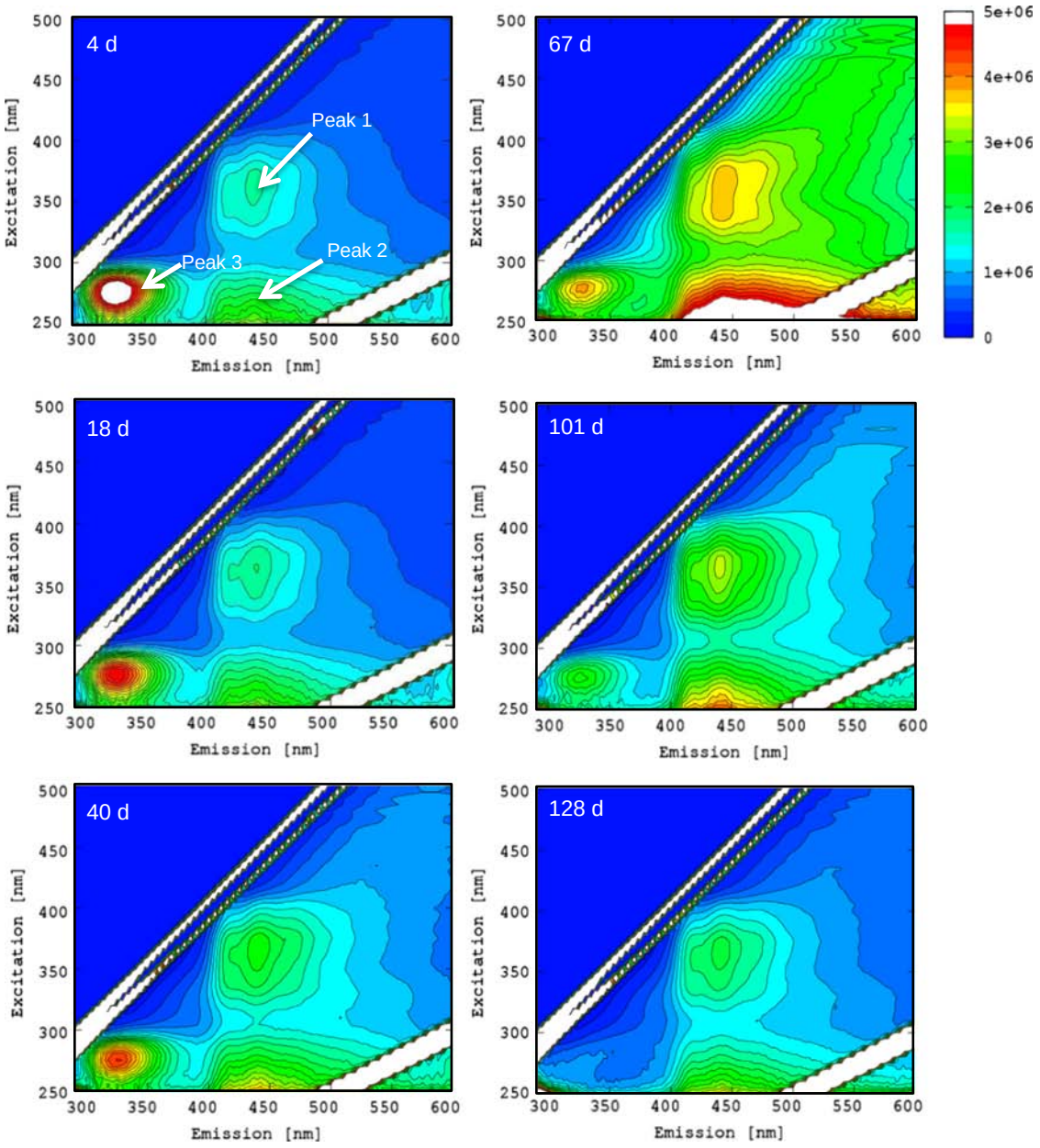

Fig. 1. Evolution of SPE-EEM spectra from a sewage sludge and green waste compost during a composting period of 128 days.

the spectra correspond to the presence of different fluorophores characterised by the excitation/emission (Ex/Em) wavelength pairs. Three peaks have been identified: peak 1 is centred at about 360/440 nm, peak 2 at 260/440 nm and peak 3 at 270/330 nm. As mentioned above, PARAFAC can decompose EEMs into various individual fluorescent components, thereby reducing the interference among fluorescent compounds [10]. Based on 32 EEMs of compost samples corresponding to the 128 days of evolution, numerous PARAFAC models were calculated using one to five components (Table 1). Explained variance and core consistency diagnostic (Corcondia) are very helpful in determining the right number of components, that is, the core consistency diagnostic helps in choosing the proper model complexity of PARAFAC models [18]. The so-called Tucker3-like core array is calculated from the data and the PARAFAC loadings [19]. A valid PARAFAC model has a core consistency close to 100% and decreases if the data cannot be described by a tri-linear model or if too many components are used [20]. In practice, the core consistency levels

Table 1
Explained variance and core consistency vs the number of components for PARAFAC models of fluorescence data with 1 to 5 components.

Number of components	Explained variance (%)	Core consistency (%)
1	83.10	100
2	98.59	99.92
3	99.51	98.54
4	99.67	35.11
5	99.83	11.20

off slowly for an increasing number of components and then sharply when the correct number of components is exceeded. The number of components corresponding to the last high consistency value must be chosen. The core consistency diagnostic scores are 99.92% for two components, 98.54% for three components, and 35.11% for four components, indicating that the three-component model provides the highest spectral resolution of components

and is likely the most appropriate model for this data set. The maximum intensities are excitation/emission values of 360/440 nm and of 250/440 nm for component 1 (C1), 290/410–470 nm for component 2 (C2), and 250/320 nm for component 3 (C3; Fig. 2). C1, C2 and C3 were very similar to peaks noticed in Fig. 1 except for C1, which included an additional fluorescence in the 240/250 nm region in addition to a high fluorescence between 350 and 370 nm compared to peak 1.

Determining relations between fluorophores by SPF-EEM and those identified in studies of WEOM remains a challenge. Despite the interest shown by liquid-phase fluorescence (LPF) on dissolved OM, SPF-EEM was very rarely used for OM characterisation in soil, compost, or solid organic waste [9,15]. Despite this lack of established references, peaks established by solid phase fluorescence present some similarities with those on WEOM/DOM fluorescence. Based on the Ex/Em values of PARAFAC components described in the literature (Table 2), C1 and C2, observed in all samples are commonly attributed to humic-like substances with high molecular weight (C1) and low molecular weight (C2) [17,21–23]. Thus, the presence of C1 indicates the formation of humic-like substances during the composting process [6]. Different molecular components derived from lignin and other degraded plant materials are potential contributors to the fluorescence at this peak. In contrast, C3 is commonly attributed to protein-like structures in the literature [23–26]. One point to note is that, after reading the interesting work of Muller et al. [9], it has been surprising not to find a limitation of fluorescence for humic-like substances (peaks 1 & 2 or component C1 and C2 in this work) as found by these authors on lignin and humic acid powders. They explained the constraint of recording fluorescence for these very dark-coloured substances by an inner filter effect, leading to a decrease of fluorescence caused by the presence of highly light-absorptive chromophores (in this case, excitation light or/and fluorescence are absorbed by chromophores).

3.2. EEM PARAFAC components behaviour

Besides the relationships based on similarities of fluorescence between DOM and solid OM, the fluorescence intensities of the three components (first loading of the PARAFAC model) also provide some additional information. However, it is important to note that

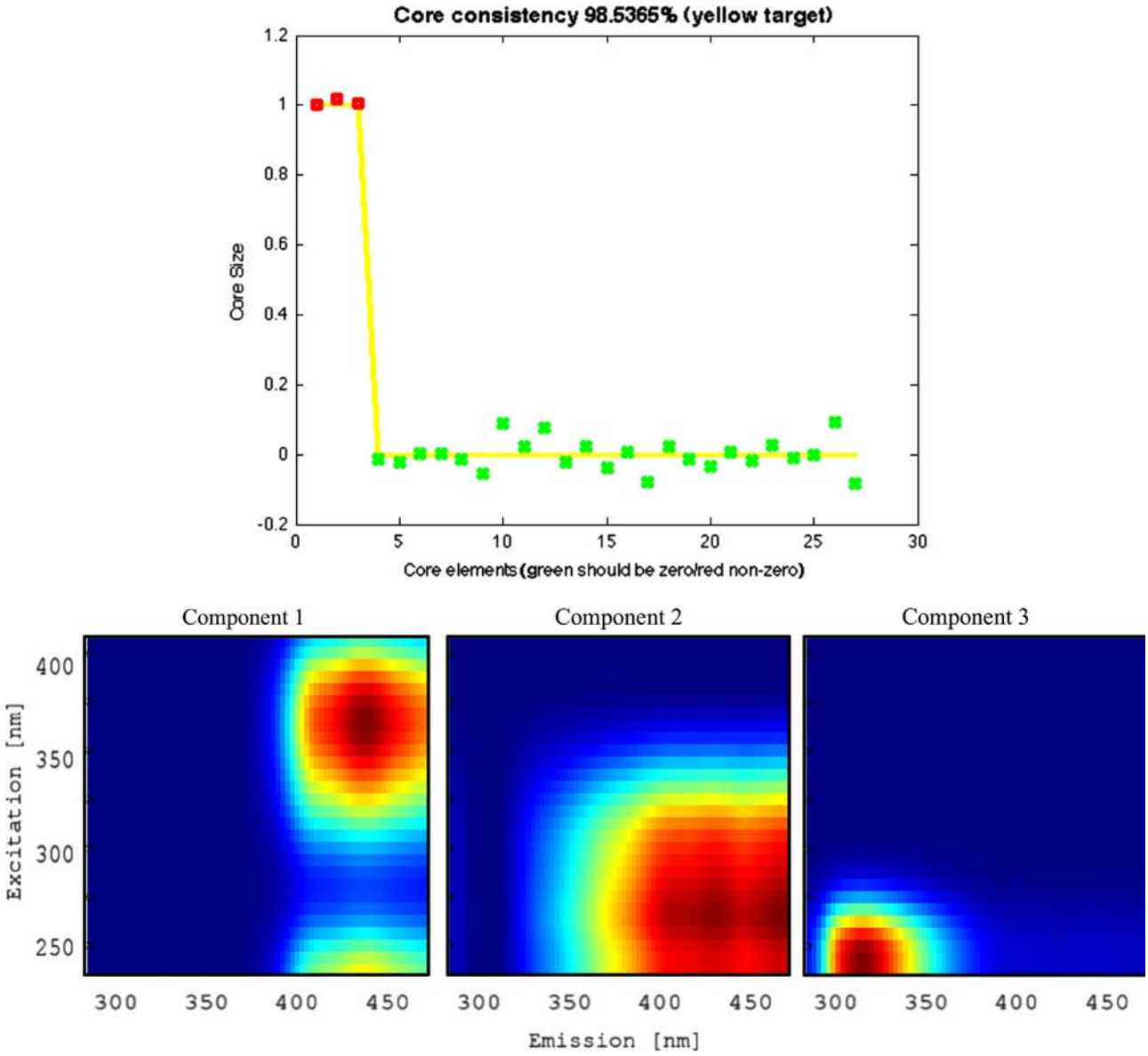

Fig. 2. Results of PARAFAC analysis with three components and 32 EEM of compost samples.

Table 2
Position of peaks (Ex/Em) and assignment of the PARAFAC derived fluorescent components according to previous fluorescence studies (mainly WEOM/DOM, adapted from Borisover et al., 2012).

Component	Position of peaks of exitation and emission (Ex/Em, nm)		Assignement
	Current study	Previous study	
1	360/440	332–448 and 245/438[a]	Humic-like, high molecular weight
	250/440	315/447[b]	
		325–330/435–440[c]	
		320–360/420–460[d]	
		320[e]/432[f]	
2	290/410–470	306/404[g]	Humic-like, low molecular weight
		< 240/465[h]	
		330/460–480[i]	
		260/400–460[j]	
		260/460[k]	
		270/480[f]	
3	250/320	270/354[b]	Protein-like stuctures
		280/330–340[i]	
		275/340[l]	
		280/325[m]	
		285/354[n]	

[a] Peaks C_C and C_A (Kothawala et al., 2012)
[b] Ohno and Bro (2006)
[c] Bertoncini et al. (2005)
[d] Peak C (Coble, 2007; Coble et al., 1998).
[e] The second exitation peak of a less intensity
[f] Borisover et al. (2012)
[g] Peak C_M (Kothawala et al., 2012)
[h] The strong excitation peak at λex < 240 nm, with a smaller and less expressed one at λex ~ 290 nm, Ohno and Bro (2006).
[i] Fellman et al. (2008)
[j] Peak A (Coble, 2007; Coble et al., 1998).
[k] Borisover et al. (2009).
[l] Peak T (Coble, 2007; Coble et al., 1998).
[m] Peak C_T (Kothawala et al., 2012)
[n] Peak T (Maie et al., 2007)

the presented relative fraction distributions are based on their relative fluorescence signal contributions, and not their contributions in terms of their respective true chemical concentrations. An expression based on the respective chemical concentrations would require knowledge on fluorescence quantum efficiencies of individual components, which are currently unknown [20]. If the identity of a PARAFAC component is unknown, it is not possible to convert fluorescence intensities to concentrations. A frequent but major mistake usually found in works since the early 2000s is the fact that authors ignore that different fluorophores can have very different efficiencies at absorbing and converting incident radiation to fluorescence [27]. One solution to obtain quantitative and qualitative information lies in the changes in the intensity of a given component, or in the ratios of any two components, between samples in the dataset. Accordingly, the use of three ratios is proposed: C1/C3, C2/C3, and C1/C2 (Fig. 3) in order to compare OM changes in each stage of composting. Both C1/C3 and C2/C3 increased steadily from 0.2 and 0.3 at 4 days to 0.9 and 0.7 after 101 days, respectively. These trends are additional evidence to the above-established links between fluorophores in liquid and solid fluorescence. Increase of humic-like substances (C1 and C2) and decrease of protein-like substances (C3) can be related to a well-known process reported above: mineralisation of easily degradable compounds (C3) and humification of OM [6,7]. C1/C2 represents a ratio between humic-like and fulvic-like substances and its increase (0.8 to 1.4) clearly reveals that the humification process occurs during the composting, as emphasised by many authors [7,28–31].

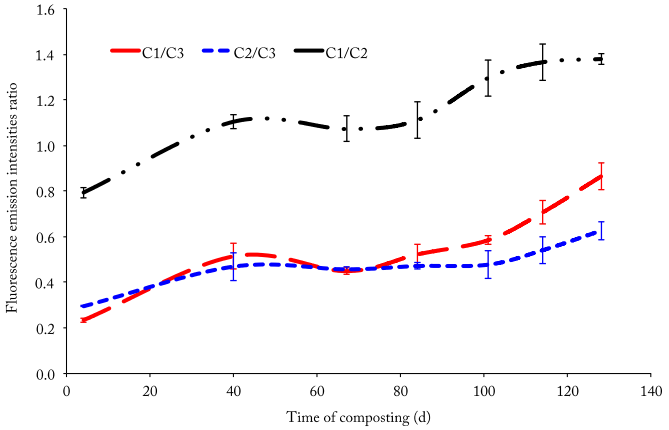

Fig. 3. Fluorescence emission intensities ratio C1/C3, C2/C3 and C1/C2 from a 3-component PARAFAC model.

Previous works using RE pyrolysis, ^{13}C CPMAS NMR, and chemical analyses on the same samples were used to support these presumptions [7,32]. Three RE pyrolysis indices were used: I-index for immature OM, R-index for the contribution of the most refractory OM and finally the ratio (A1 + A2)/(A3 + A4) for the hydrocarbon compounds released between 205–400 °C and 400–550 °C. Solid-state ^{13}C CPMAS NMR was also used with the following dominant forms of carbon based on their ^{13}C chemical shifts: alkyl C, for example, from amino acids, lipids and waxes (0–45 ppm), O-alkyl C, for example, from cellulose and hemicelluloses (45–110 ppm), aromatic C (110–145 ppm) and phenolic C, for example, from lignin (145–165 ppm), and finally carbonyl–carboxyl C (165–210 ppm). Pearson's correlation coefficients were calculated using computed EEMs of PARAFAC components and several indices of OM changes (Table 3). Significant figures (*p-value* < 0.05) are presented in bold. The main significant and most interesting ($r > 0.5$) are the following:

- C1/C3 and C2/C3 are significantly and positively correlated with R-index but significantly and negatively correlated with I-index and (A1 + A2)/(A3 + A4) ratio
- C1/C3 and C2/C3 are significantly and positively correlated with ^{13}C Aromaticity
- C1/C3 and C2/C3 are significantly and negatively correlated with OM/(HA + FA) ratio
- C1/C2 is significantly and positively correlated with HA/FA ratio

Hence, positive correlations between both C1/C3, C2/C3, and the R-index (refractory OM) but negative correlation with the I-index (immature OM) reveal a decrease in the labile part of the OM in the compost and an increase in the humic-like substances, as showed by Albrecht et al. [32]. These significant links between PyRE and SPF-EEM results are additional indications that PARAFAC applied to SPF-EEM is useful to monitor OM changes. Further support is given by the significant and negative correlations between both C1/C3 and C2/C3 and PyRE (A1 + A2)/(A3 + A4) ratio. The (A1 + A2)/(A3 + A4) ratio represents a relation of labile (A1) and resistant biopolymers (A2) versus immature geopolymers and refractory fraction (A3 + A4), that is, humic-like substances. Then, an increase of both, the C1/C3 and the C2/C3 ratios, as well as a decrease in (A1 + A2)/(A3 + A4) ratio, revealed humification processes as well, occurring during composting.

Comparison of SPF-EEM parameters with ^{13}C NMR ones provides similar results. ^{13}C Nuclear Magnetic Resonance Spectroscopy in the solid state (^{13}C CPMAS NMR) is often used as a reference tool to collect direct information on structural characteristics of OM in composts, soil, peats, etcetera. [33,34]. According to Vinceslas-Akpa and Loquet [35], aromaticity provides an overall

Table 3
Pearson's correlation coefficients between EEM PARAFAC components fluorescence ratios and three RE pyrolysis indices (I-index for immature OM, R-index for most refractory OM and the ratio (A1+A2)/(A3+A4) for the hydrocarbon compounds released between 205–400 °C and 400–550 °C), OM aromaticity by ^{13}C CPMAS NMR, and chemical parameters (HA=humic acid, FA=fulvic acid). Bold numbers: significant correlation ($p < 0.05$).

	C1/C3	C2/C3	C1/C2	R-Index	I Index	(A1+A2)/(A3+A4)	Aromaticity (13C NMR)	OM / (HA+FA)	HA/FA
C1/C3	1	**0.78**	**0.68**	**0.86**	**−0.92**	**-0.88**	**0.66**	**−0.71**	0.47
C2/C3		1	0.15	**0.61**	**−0.67**	**-0.63**	**0.57**	**−0.5**	0.24
C1/C2			1	0.68	−0.69	−0.68	0.48	−0.6	**0.67**
R-Index				1	**−0.94**	**-0.99**	**0.58**	**−0.87**	0.68
I-Index					1	**0.94**	**−0.54**	**0.8**	−0.61
(A1+A2)/(A3+A4)						1	**−0.64**	**0.88**	−0.7
Aromaticity (13C NMR)							1	**−0.64**	0.6
OM / (HA+FA)								1	−0.83
HA/FA									1

view of the evolution of aromatic compounds and enables the degree of humification in compost, in terms of the accumulation of aromatic compounds, to be characterised. Hence, simultaneous increases of both C1/C3 and C2/C3 and C aromaticity measured by ^{13}C NMR on the same samples [7], both revealed humification of OM. Therefore, with significant and positive correlation coefficients, ^{13}C NMR gives a precious validation of SPF-EEM to monitor OM changes. Humification of OM is also confirmed by significant and negative correlations of both C1/C3 and C2/C3 and increase of humic-like substances during composting process that is, OM/(HA+FA) ratio.

Recent studies underline the importance of ecosystem properties in soil organic matter (SOM) stabilisation processes, such as physical disconnection between SOM and microbes or organo-mineral associations [36]. This is indeed supported by progress in analytical and visualisation techniques. Methods used to classify soil OM into active, slow, and passive pools are no longer chemically, but rather physically performed, based on the various degrees of physical protection of SOM by either aggregates, association force between SOM and minerals, or different particle sizes [37]. However, in compost science, the ecosystem properties of OM remain totally different and determining humic-like substance contents is a frequently-used tool to assess the degree of maturity during the composting process [38,39]. That is why significant and positive correlations between HA/FA ratio and C1/C2 (r=0.67) give additional evidence that SPF-EEM is capable of providing information about changes of OM during the composting process. An increase of HA/FA ratio can indeed be monitored by their specific fluorescence. Compost is a pertinent model to study OM evolution (mineralisation and humification processes) with fairly clear and well-documented transformations of OM, without any complex "interferences" due to pedogenetic processes occurring in soils. The next step of such an approach is its application to more complex samples such as, indeed, soil samples. In addition, as noticed by Milori et al. [16] and Gonzales-Perez et al. [40], another advantage of fluorescence spectroscopy, and thereby possibly SPF-EEM, is that it can be used when it is not possible to obtain reliable Electron Paramagnetic Resonance (EPR) and NMR spectra because of interferences from iron oxide, minerals, and other paramagnetic ions, as found in oxisol samples.

4. Conclusions

SPF-EEM is a promising technique for monitoring OM during the evolution of compost processes. Furthermore, SPF-EEM is a valuable approach to a rapid and accurate assessment of the degree of OM transformation. It is sensitive and allows rapid analysis of samples without any prior chemical treatment. The sensibility of SPF-EEM allows identification of the main/common protein-like and humic-like fluorophores. Their signature is related to an increase in maturity due to changes in chemical composition, and a decrease of more easily degradable compounds.

Acknowledgements

We acknowledge R. Redon and S. Mounier (PROTEE laboratory, Univ. Toulon, France) for providing their Progmeef MatlabTM toolbox. The authors would like to thank the anonymous reviewers for their valuable comments and suggestions to improve the quality of the article.

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