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A comparison of extraction procedures for water-extractable organic matter in soils

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Summary

The characteristics of dissolved organic matter (DOM) in soils are often determined through laboratory experiments. Many different protocols can be used to extract organic matter from soil. In this study, we used five air-dried soils to compare three extraction methods for water-extractable organic matter (WEOM) as follows: (i) pressurised hot-water-extractable organic carbon (PH-WEOC), a percolation at high pressure and temperature; (ii) water-extractable organic carbon (WEOC), a 1-hour end-over shaking; and (iii) leaching-extractable organic carbon (LEOC), a leaching of soil columns at ambient conditions. We quantified the extraction yield of organic carbon; the quality of WEOM was characterized by UV absorbance, potential biodegradability (48-day incubation) and parallel factor analysis (PARAFAC) modelling of fluorescence excitation emission matrices (FEEMs). Biodegradation of dissolved organic carbon (DOC) was described by two pools of organic C. The proportions of labile and stable DOC pools differed only slightly between the WEOC and LEOC methods, while PH-WEOC contains more stable DOC. The mineralization rate constants of both labile and stable DOC pools were similar for the three methods. The FEEMs were decomposed into three components: two humic-like fluorophores and a tryptophan-like fluorophore. The effect of extraction method was poorly discriminant and the most similar procedures were PH-WEOC and LEOC while WEOC extracts were depleted in humic-like fluorophores. This study demonstrates that WEOM quality is primarily determined by soil characteristics and that the extraction method has a smaller, but still significant, impact on WEOM quality. Furthermore, we observed considerable interaction between extraction procedure and soil type, showing that method-induced differences in WEOM quality vary with soil characteristics.

Introduction

Mineralization and humification are major processes controlling the evolution of soil organic matter (SOM) in soil horizons and are closely related to dissolved organic matter (DOM) degradation and/or production. As DOM is the most active and mobile form of SOM (Zech *et al.*, 1997; Corvasce *et al.*, 2006), an understanding of its dynamics is important in order to evaluate better the role of soils in the terrestrial carbon (C) cycle. The concentration of DOM in the soil solution is regulated by the balance between production, degradation, stabilization and leaching (Kalbitz *et al.*, 2000). The production of DOM is mainly controlled by biological processes, such as the decomposition of SOM, the release of root exudates and the lysis of microorganisms (Kalbitz *et al.*, 2000). The DOM represents a source of energy and nutrients for soil ecosystems and is the direct precursor of microbial growth and activity. It is thus the main driver for the decomposition processes of organic matter in soil (Boyer & Groffman, 1996; Marschner & Kalbitz, 2003).

Concentrations and fluxes of DOM in soils are often determined in laboratory experiments by characterizing water-extractable organic matter (WEOM). Many extraction protocols have been used to extract organic matter from soil (Wagai & Sollins, 2002; Kalbitz *et al.*, 2003; McDowell *et al.*, 2006), which complicates inter-study comparison. Such protocols diverge in terms of pressure, temperature (up to 200°C), extraction duration (from a few minutes to several hours), type and proportion of extractant and type of extraction (including leaching, end-over shaking or soil suspension). This variety of protocols could therefore lead to differences in the results obtained for extraction yield and WEOM quality (Schwesig *et al.*, 1999; Wagai & Sollins, 2002; Landgraf *et al.*, 2006).

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The implications of these potential differences have rarely been studied, and have been limited to a single soil type in any given study (Schwesig *et al.*, 1999; Zaccone *et al.*, 2009; Nkhili *et al.*, 2012).

In this study, we compare three different extraction procedures (high pressure and temperature, shaking and leaching) by quantifying and characterizing the WEOM from five different soils, all collected in France. The main objective was to determine the effect of each method on WEOM quality by measuring its potential biodegradability, absorbance and fluorescence properties.

Materials and methods

Sampling design

The three extraction procedures were compared, using samples from the A-horizon of five different soil types (IUSS Working Group WRB, 2006) developed on different parent materials, with various vegetation types, all from France. The sampling areas were all located within clearly identified soil units. The soils were selected with particular attention to texture and organic C content, to ensure that various types of organo-mineral associations would be studied. At each site, a 5-kg composite of the A-horizon was collected from a soil pit with well-defined horizons. All soils were air-dried, sieved through a 2-mm mesh and homogenized before further experiments. Nine samples were randomly taken from each of the five soil composites. We then allocated, for each of the five soils, three of these nine samples to one of the three extraction methods (see below), thus giving a balanced design (five soils × three replicates × three methods) with 45 observations. For the biodegradation experiment and the fluorescence analysis, all measurements were performed on each of the 45 extracts.

Soils

Soil 1. DA is a Dystric Andosol collected from 'Le Puy de la Vache' in the Auvergne region (45°40′37″N; 2°57′53″E), on the eastern slope of a Holocene scoria cone, at an elevation of 1032 m above sea level. It is an organic-rich soil, characterized by a small bulk density and the presence of allophanes. Vegetation is transitional woodland, mainly composed of conifers and ferns.

Soil 2. EP is an Entic Podzol collected from the top of 'Le Massif de la Serre' in the Franche-Comté region (47°11′29″N; 5°33′50″E), at an elevation of 378 m above sea level. The bedrock is Buntsandstein siliceous sandstone (Lower Triassic). This soil is well-drained and a thick, acidic mor-type organic horizon is present. Vegetation is a broad-leaved forest composed of oak, hornbeam and beech.

Soil 3. DC is a Dystric Cambisol collected in the Burgundy region (47°06′16″N; 4°25′55″E). The site is located 390 m above sea level, near the top of a slightly sloping topographic depression. Vegetation is a mainly gramineous pasture, and bedrock consists of Upper Triassic siliceous sandstone.

Soil 4. EC is a Eutric Cambisol collected in the Burgundy region (47°23′12″N; 4°39′19″E). The site is located 361 m above sea level, at the bottom of a valley, on calcareous alluvial deposits surrounded by Jurassic carbonate rocks. Vegetation is a mainly gramineous pasture.

Soil 5. GL is a Gleyic Luvisol collected from an agricultural area in the Burgundy region (47°07′23″N; 5°05′08″E). The site is located 196 m above sea level, in the Bressan Graben and the parent material is recent loam deposits (Plio-Pleistocene). The soil has a massive structure and was bare when sampled.

Organic C and N contents were measured by dry combustion (Vario MICRO cube, Elementar, Hanau, Germany) after inorganic C removal from carbonate-containing samples by addition of an excess of 2 M HCl. Soil pH was determined in soil suspension (soil:water ratio of 1:4). Soil texture was determined using the Robinson pipette method and total carbonate content was quantified by the volumetric method using a Bernard calcimeter according to the standard French procedures (AFNOR, 1983, 1995). All measurements were performed in triplicate and characteristics for each soil are reported in Table 1.

Extraction procedures

Extraction method 1. Pressurised hot-water-extractable organic carbon (PH-WEOC) was extracted using a solvent extractor (ASE200, Dionex Corporation, Sunnyvale, California, USA). A 10-g soil sample was thoroughly mixed with 10 g of acid-washed sand to enhance the drainage capacity of the soil. This mixture was placed in an extraction cell with a capacity of 22 ml and glass beads were added to completely fill the cell. Extraction consisted of five steps: (i) the cell was filled with water; (ii) pressure and temperature were stabilized at 100°C and 10 MPa, respectively, over a 5-minute period; (iii) the extractable organic matter was dissolved at 100°C and 10 MPa during the static phase, which lasted for 10 minutes; (iv) the cell was flushed with 13 ml of ultrapure water; and (v) the cell was purged with an inert gas (N_2) for 2 minutes. All conditions were based on the study of Schwesig *et al.* (1999), who have shown that WEOM was not altered at these settings. Before further analyses, all extracted solutions were filtered through 0.45-µm pore-size cellulose acetate filters.

Extraction method 2. Water-extractable organic carbon (WEOC) was obtained by shaking a 50-g soil sample with 200 ml ultrapure water at 120 revolutions per minute for 60 minutes at room temperature. Extraction solutions were then centrifuged for 15 minutes at 4600 ***g*** and filtered through 0.45-µm pore-size cellulose acetate filters. Unlike the two other methods, the WEOC extraction method led to the breakdown of soil aggregates.

Extraction method 3. Leaching-extractable organic carbon (LEOC) extractions were carried out on 100-g soil samples placed in glass columns (inner diameter = 54 mm). Glass beads were placed on the top to allow a homogeneous distribution of water

Table 1 Organic C and N contents, texture and pH of soils analysed for the comparison of extraction procedures

Soil	Classification WRB 2006	C (mg g^{-1})	N (mg g^{-1})	C/N	$CaCO_3$ (mg g^{-1})	Sand (%)	Silt (%)	Clay (%)	pH
DA	Dystric andosol	135.9 (1.6)	8.60 (0.23)	15.8 (0.3)	–	31.5 (0.2)	47.3 (0.3)	21.2 (0.1)	5.49 (0.05)
EP	Entic Podzol	65.9 (0.4)	2.91 (0.01)	22.7 (0.2)	–	70.2 (0.4)	18.8 (0.1)	11.0 (0.1)	4.11 (0.02)
DC	Dystric Cambisol	29.7 (0.3)	2.88 (0.01)	10.3 (0.1)	–	58.5 (0.3)	21.6 (0.1)	19.9 (0.1)	5.29 (0.05)
GL	Gleyic Luvisol	9.6 (0.1)	1.00 (0.02)	9.4 (0.1)	–	22.3 (0.1)	64.6 (0.3)	13.1 (0.1)	5.98 (0.05)
EC	Eutric Cambisol	24.9 (0.2)	2.82 (0.03)	8.8 (0.1)	193 (3)	9.0 (0.1)	57.7 (0.3)	33.3 (0.2)	7.58 (0.05)

Values are means of three replicates and standard errors are given in parentheses.

over the section. Acid-washed sand was placed at the bottom of the column to prevent clogging and loss of soil particles. Columns were moistened overnight to avoid the creation of preferential flow paths. Each column was leached at room temperature with 400 ml ultrapure water at a flow rate of 1.5 ml minute^{-1}. The percolated solutions containing LEOC were collected and filtered through 0.45-µm pore-size cellulose acetate filters.

For each of the five soils, the three extraction methods were repeated three times on different samples, thus giving 45 observations (five soils × three replicates × three methods). During subsequent steps, all measurements were performed on each of the 45 extracts.

Preliminary measurements

The extraction volume was determined gravimetrically and the pH was measured with a microelectrode N 5800 A (Schott Instruments, Mainz, Germany). The ultraviolet (UV) absorbance at 254 nm was measured with a Jenway 6715 spectometer (Stone, Staffordshire, England). The specific UV absorbance at 254 nm ($SUVA_{254}$) of the samples was calculated as follows:

$$SUVA_{254} = \frac{UV_{254}}{b \times C}, \quad (1)$$

where UV_{254} is ultraviolet absorption at 254 nm, b is the optical path length in metres and C is the DOC concentration of the samples in milligrams per litre.

Organic C content in the extracted solutions was quantified in 5-ml subsamples with a total organic carbon analyser (TOC 5000A, Shimadzu, Kyoto, Japan), after the addition of 20 µl 2 M HCl to remove inorganic C. The extraction ratio (ER), expressing the yield of the extraction procedure, was calculated as the ratio of extracted C to soil total organic C.

Determination of dissolved organic carbon biodegradability

The biodegradability of DOC was assessed by incubating the extracted solutions in the dark at 21°C for 48 days, with regular shaking. After a 0.22-µm membrane-filtration (cellulose nitrate) to remove microorganisms, solutions were diluted to a final concentration of 20 mg C l^{-1} or less, to avoid excessive microorganism growth. Sixty millilitres of each DOC solution were transferred to 145-ml incubation flasks. We then added 1 ml of a nutrient solution (ionic strength = 0.12 M), prepared with $(NH_4)_2SO_4$ and KH_2PO_4 to ensure C:N:P:S:K molar ratios of ≤ 5:1:1:1:1 (Bowen *et al.*, 2009). To obtain a broad microbial diversity (Kalbitz *et al.*, 2003), an inoculum was prepared from a mixture of the five soils and added to each sample, just before the flasks were sealed and incubated. A glucose solution (20 mg C l^{-1}) and an ultrapure water solution, each with the addition of the nutrient solution and the inoculum, were used as controls.

The CO_2 concentration was measured in the headspace of the flasks on days 1, 3, 5, 7, 14, 28 and 48, using gas chromatography and thermal conductivity detection (7890A, Agilent, Santa Clara, California, USA). The molar amount of CO_2 in the gas phase was calculated using the ideal gas law equation, and the calculation of CO_2 in the liquid phase was based on Henry's law. The sum of these two pools was considered as the total amount of CO_2 produced by microbial degradation. Mean values measured for the controls (ultrapure water) were deducted from values for the DOC-containing flasks. To analyse the kinetics of DOC mineralization in the incubation experiment, following Kalbitz *et al.* (2003), a double exponential model with two distinct DOC pools was fitted to the calculated amount of produced CO_2 by means of the Quasi-Newton least-squares optimization method, using the maxLik package (Toomet & Henningsen, 2012) in R software (R Core Team, 2012). The double exponential mineralization curve was of the form:

$$\text{Mineralized DOC} = (100 - a)\left(1 - \exp^{-k1t}\right) + a\left(1 - \exp^{-k2t}\right), \quad (2)$$

where mineralized DOC is the CO_2 produced in per cent of initial DOC, t is time in days, $(100 - a)$ is the percentage of DOC that is rapidly mineralized (labile pool), a is the percentage of DOC that is slowly mineralized (stable pool), $k1$ is the mineralization rate constant of the labile pool of DOC and $k2$ is the mineralization rate constant of the stable pool of DOC (both expressed per day).

Fluorescence analysis and PARAFAC modelling

For 3D-fluorescence spectroscopy measurements, water-extracted solutions were diluted with ultrapure water in order to reach UV_{254} of 0.1. The fluorescence excitation emission matrices (FEEMs) were obtained with a Hitachi F-4500 (San Jose, California, USA)

with the following settings: speed scan, 2400 nm minute^{-1}; excitation and emission bandwidth, 5 nm; excitation and emission step, 5 nm; response, 0.1 s; excitation interval, 250–500 nm; and emission interval, 250–600 nm. The FEEMs were corrected for inner-filter effects following the controlled dilution approach (Luciani *et al.*, 2009). The 45 FEEMs were numerically corrected for Rayleigh and Raman scattering peaks before parallel factor analysis (PARAFAC) treatment. The PARAFAC treatment of FEEMs is well referenced (see Coble, 1996; Stedmon & Bro, 2008) and will not be detailed in this paper. Briefly, the 45 FEEMs were computed to identify the fluorescent components present in samples and their relative contribution to total fluorescence (Luciani *et al.*, 2008) using the alternating least square algorithm of the 'N-way toolbox for MATLAB' (Andersson & Bro, 2000). The most appropriate number of components was assessed by using the core-consistency diagnostic (CORCONDIA) score. A three-component model, explaining 98.9% of FEEM variability and giving a CORCONDIA score of 81.7%, was selected as the appropriate PARAFAC model.

Statistical data analysis

Two-way ANOVAs were computed to estimate the effect of soil, of extraction method and of their interaction, on extraction ratio (ER), pH, $SUVA_{254}$, biodegradation characteristics and contribution scores of PARAFAC components. When necessary, we applied log transformations to satisfy the requirements of residual normality and variance homogeneity. Our interest lies in the pairwise differences between the three extraction methods, as none was considered as the reference method. These simple pairwise comparisons of the three methods were therefore assessed using the least significant difference test (LSD, $P \leq 0.05$), once their main effect had been found to be significant in the ANOVAs. The nine WEOM samples obtained from the EC soil were excluded from the ANOVAs for biodegradation parameters and PARAFAC components because preliminary tests showed that the EC results biased the ANOVAs for these variables. The values obtained for the EC soil for these variables are nevertheless presented and discussed in the following sections.

A principal component analysis (PCA) was performed on the correlation matrix of the DOC quality characteristics. The plot of the PCA individual scores on the first two components shows graphically the reproducibility of the methods. This reproducibility was numerically assessed with respect to the sum of the variances of the extraction triplicates on all principal components. All computations used JMP 9.0 (SAS Institute) statistical software.

Results and discussion

Extraction ratio, pH and $SUVA_{254}$

We observed great variability in extraction ratios (ERs), ranging from 2.1 ± 0.2 mg C g^{-1} (mean ± standard error of the mean) to 35.6 ± 0.5 mg C g^{-1} soil C, depending on soils and extraction procedures (Figure 1). For all methods, the ERs measured were of the same order of magnitude as in other studies using comparable extraction procedures (Schwesig *et al.*, 1999; Gregorich *et al.*, 2003; Akagi *et al.*, 2007). The two-way ANOVA revealed a significant effect of both extraction procedure and soil on ER (Table 2). For all three methods, the largest ERs were found for soils GL and EP, and the smallest were found for EC and DA. The PH-WEOC method had the largest extraction yields, with ERs larger by a factor of 2–12 than the two other methods (Figure 1). A positive correlation between WEOM content and CO_2 fluxes was reported in Zhao *et al.* (2008). These authors measured about four times more C mineralized as CO_2 after 35 days than C in WEOM at the initial stage, suggesting that a large proportion of potentially biodegradable C was not extracted at the initial stage. In our study, therefore, the increase in ER with the PH-WEOC method could correspond to the extraction of a potentially reactive organic pool. The smaller ERs for the WEOC and LEOC methods were in similar ranges, and values were consistent for all soils (Figure 1). The effect of interaction between extraction procedure and soil was also significant, meaning that the impact of the extraction procedure varied from one soil to another (Table 2). This interaction is clearly apparent for soil EC, which had the smallest ERs with the WEOC and LEOC methods and an intermediate ER with PH-WEOC (Figure 1). The ER depends both on the extraction procedure and on the soil characteristics (such as texture, organic matter content and quality and/or type of aggregation). This result confirms the relevance of using several different soils to compare extraction procedures.

The pH measurements showed that parent material was the major influence on the chemistry of the extracted solutions, with smaller values for DA, EP and DC, which are all soils developed on

Table 2 Summary table for the two-way ANOVAs of extraction ratio (ER), pH and $SUVA_{254}$

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Log(ER)					
Method	2	16.056	8.02788	7929.5	< 0.05
Soil	4	8.598	2.14957	2123.2	< 0.05
Method:soil	8	2.556	0.31952	315.6	< 0.05
Residuals	27	0.027	0.00101	–	–
Total	41	26.508			
pH					
Method	2	4.574	2.2871	191.0	< 0.05
Soil	4	70.983	17.7457	1482.1	< 0.05
Method:soil	8	1.871	0.2338	19.5	< 0.05
Residuals	30	0.359	0.0120	–	–
Total	44	77.787			
Log($SUVA_{254}$)					
Method	2	1.126	0.5631	377.9	< 0.05
Soil	4	1.793	0.4482	300.9	< 0.05
Method:soil	8	2.316	0.2895	194.3	< 0.05
Residuals	28	0.042	0.0015	–	–
Total	42	5.271			

We excluded three observations for ER and two observations for $SUVA_{254}$, in order to fit the ANOVA model correctly on our data.

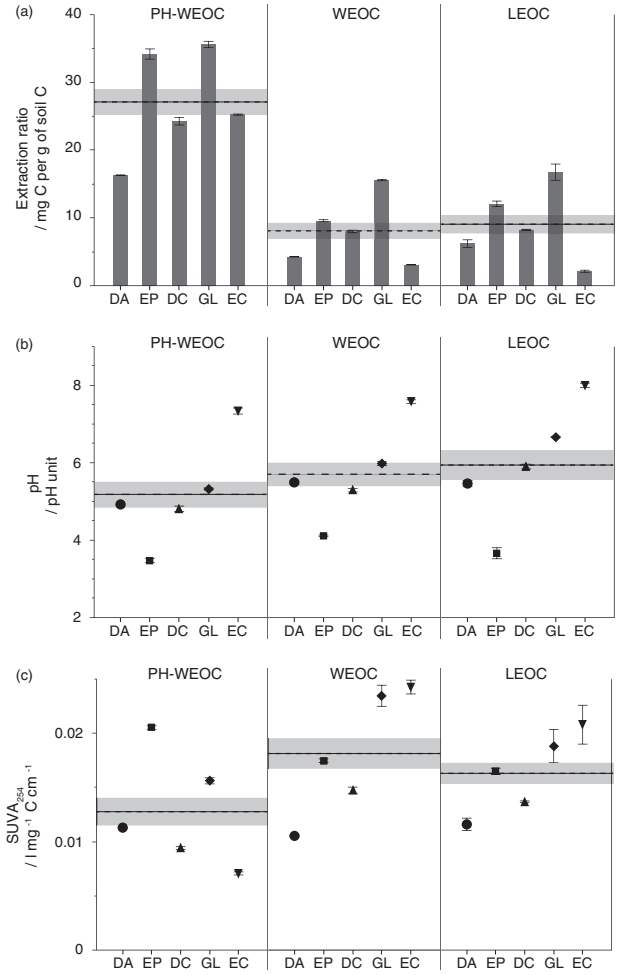

Figure 1 (a–c) Extraction ratio (ER) in mg C extracted per gram of soil organic carbon (SOC), pH and specific UV absorbance at 254 nm ($SUVA_{254}$) in $l\,mg^{-1}\,C\,cm^{-1}$. Error bars represent the standard error of the mean of the three replicates. For each method, the dashed line and grey band represent the mean and the standard error of the mean.

siliceous rocks, while the largest pH values were measured for EC, a soil developed on calcareous rocks (Figure 1). We also observed different pH values for the extracts from the three methods. The PH-WEOC extracts were more acidic, with pH values between 0.5 and 1.3 pH units lower than the other two methods. These lower pH values are attributed to the greater organic C concentrations in the extracted solutions, as observed in the study by Nkhili *et al.* (2012). The statistical analysis confirmed the major effect of soil on pH and the less pronounced, but considerable, effect of extraction method (Table 2).

The values of specific UV absorbance at 254 nm ($SUVA_{254}$) for extracted solutions ranged from 0.007 to 0.024 $l\,mg^{-1}\,C\,cm^{-1}$ (Figure 1) and the ANOVA revealed the significant effects of soil, of method and of their interaction on $SUVA_{254}$ (Table 2). We measured smaller $SUVA_{254}$ values for DA and DC soil extracts, while there were larger values in extracts from EP and GL soils, indicating greater aromatic C contents (Chin *et al.*, 1994; Weishaar *et al.*, 2003). The PH-WEOC extracts were less aromatic, suggesting that the increase in ER with PH-WEOC corresponds to the solubilization of less aromatic organic compounds, such as carbohydrates or organic acids. The smaller $SUVA_{254}$ values could also result from the alteration of aromatic organic molecules during extraction, as suggested by Nkhili *et al.* (2012) for hot-water extraction performed at 60°C. However, Schwesig *et al.* (1999) established with NMR measurements that, up to 150°C, WEOM was not altered with the PH-WEOC method. The biodegradation of labile organic compounds, such as carbohydrates and amino acids, may have occurred during the LEOC and WEOC extractions (Rousk & Jones, 2010), which take more time and were conducted at ambient conditions. This process would not occur during PH-WEOC extraction because the extraction conditions of temperature and pressure must inhibit biodegradation. The preservation of labile compounds might thus explain the smaller $SUVA_{254}$ values measured in PH-WEOC extracts.

The $SUVA_{254}$ values for PH-WEOC extracts were smaller for DC and GL, but larger for EP, while the aromaticity of DA remained unchanged (Figure 1). The EC soil gave contrasting results, with the largest $SUVA_{254}$ values for WEOC and LEOC and the smallest for PH-WEOC. These differences in the effect of method in relation to soil highlight the significant interaction between the extraction method and the soil, suggesting that generalizations about method-induced effects are not without risk.

Biodegradation experiment

The CO_2 produced from the glucose solution after the 48-day incubation confirmed that microorganism activity was adequate, with $85.7 \pm 2.3\%$ of the initial glucose C mineralized. For soil extract samples, the proportion of mineralized C ranged from 45 to 100% of the initial DOC stock and no lag phases were detected. It must be noted that the standard errors for biodegradation characteristics were large, confirming the limited reproducibility of such experiments, as observed in other studies (Kalbitz *et al.*, 2003; Kiikkilä *et al.*, 2005). This type of experiment is based on the colonization of a sterile medium by microorganisms, and slight differences at the initial stage of incubation may result in divergence in microbial community composition and establishment. For this reason, the double exponential model was fitted to each replicate.

The good fit of the model (all fitted curves had $R^2 > 0.96$) confirms that DOC mineralization was adequately described when considering two pools of organic C: a readily decomposable labile pool, and a stable pool that was more resistant to mineralization. The labile pool accounted for 15.5–41.2% of total DOC in our samples, with mineralization rate constants ranging from 0.17 to 1.07 day^{-1}. For the stable pool, which accounted for 58.8–84.5% of total DOC, the mineralization rate constants ranged from 0.007 to 0.048 day^{-1} (Figure 2). The EC extracts are unusual and will be discussed after the main trends. As the values obtained for the EC extracts were so atypical, they masked the main trends in the data, and were therefore removed from the ANOVA for stable DOC (a) and mineralization rate constants ($k1$ and $k2$).

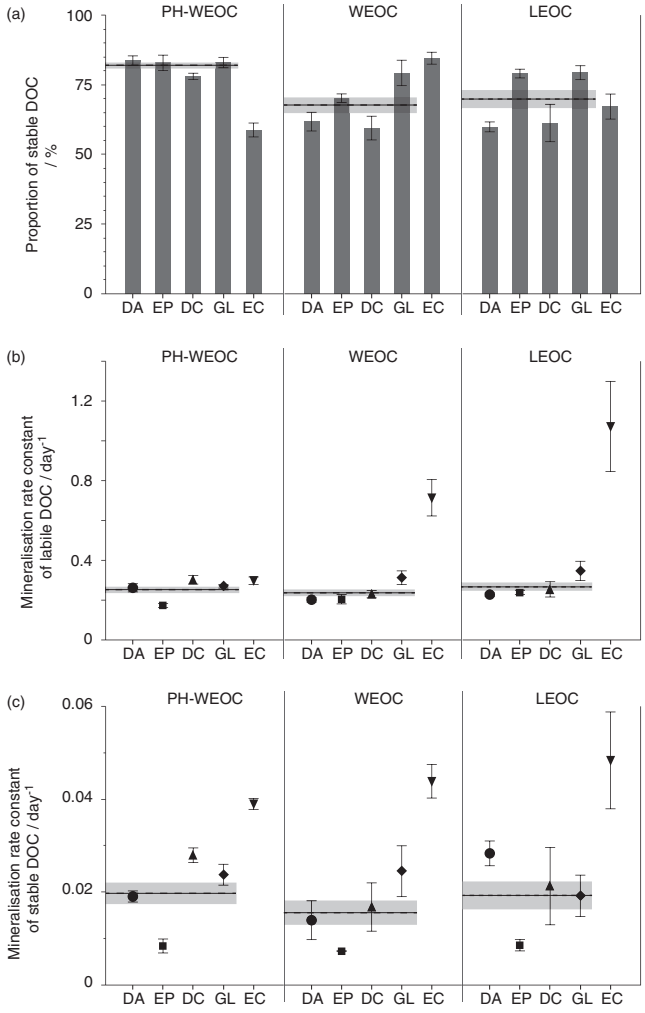

Figure 2 (a–c) Percentage of stable DOC pool (*a*) and mineralization rate constants of labile (*k1*) and stable (*k2*) pools, both expressed on a per day basis. Error bars represent the standard error of the mean of the three replicates. For each method, the dashed line and grey band represent the mean and the standard error of the mean. The values for the EC soil were not included in the calculation of the means and the standard error of the means.

The two-way ANOVA indicated significant effects of method, of soil and of their interaction on the size of the stable pool (Table 3). The stable pool was large for solutions obtained from soils EP and GL while DC gave solutions with smaller proportions of stable DOC (Figure 2). The proportions of stable DOC were consistent for samples obtained by either the LEOC or the WEOC method. For all these samples, we found a good correlation between the aromaticity ($SUVA_{254}$) and the size of the stable pool ($r = 0.66$; $n = 30$). Relationships between poor biodegradability and high aromaticity of WEOM have already been reported in other studies (Kalbitz *et al.*, 2003; McDowell *et al.*, 2006; Zhao *et al.*, 2008). The PH-WEOC extracts were more enriched in stable DOC than extracts from the two other methods. This result is contradictory, as these extracts were previously characterized by small $SUVA_{254}$ values.

Table 3 Summary table for the two-way ANOVAS of the biodegradation characteristics

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
a					
Method	2	1403.9	701.9	22.53	< 0.05
Soil	3	1274.9	425.0	13.64	< 0.05
Method:soil	6	552.3	92.1	2.95	< 0.05
Residuals	24	747.9	31.2	–	–
Total	35	3978.9			
k1					
Method	2	0.0053	0.0026	1.45	0.25
Soil	3	0.0545	0.0181	9.96	< 0.05
Method:soil	6	0.0237	0.0040	2.17	0.08
Residuals	24	0.0438	0.0018	–	–
Total	35	0.1272			
k2					
Method	2	0.00012	0.000062	1.36	0.27
Soil	3	0.00126	0.000420	9.17	< 0.05
Method:soil	6	0.00044	0.000073	1.59	0.19
Residuals	24	0.00110	0.000046	–	–
Total	35	0.00292			

a is the percentage of stable DOC, *k1* is the mineralization rate constant of labile DOC and *k2* is the mineralization rate constant of stable DOC.
The nine observations corresponding to the EC soil were excluded from the dataset because preliminary tests revealed that these samples biased the ANOVA models.

Therefore the additional amounts of DOC extracted with this method preferentially originated from a more recalcitrant C pool that would release organic compounds with less aromaticity. The EC extracts produced very different results, with a smaller proportion of stable DOC in the PH-WEOC extracts, but much more in the WEOC extracts (Figure 2), consistent with the $SUVA_{254}$ values.

The mineralization rate constants of the labile (*k1*) and stable (*k2*) pools give additional indications about DOC pool biodegradability. We detected no effect of the extraction procedure on *k1* (Table 3). For soils DA, EP, DC and GL, values of *k1* ranged from 0.17 to 0.35 day^{-1} (Figure 2), and decreased in the following order: GL > DC > DA > EP. For soil EC, *k1* ranged from 0.30 to 1.07 day^{-1} and greatly different values were obtained for each method.

The mineralization rate constants of the stable pool (*k2*) ranged from 0.007 to 0.048 day^{-1} (Figure 2). For the three methods, stable DOC from soil EP had the smallest *k2*, while the largest values were obtained for soil EC. The values obtained for DA, DC and GL were close, and were all intermediate. No significant differences in *k2* were induced by the extraction procedure, nor by interaction (Table 3), revealing similar potential for biodegradation of the stable pool of DOC, whatever the method used.

The *k2* parameter was strongly correlated with the pH of the solution extracted ($r = 0.76$; $n = 45$), suggesting that pH controls the biodegradation of this large pool of DOC. This correlation may

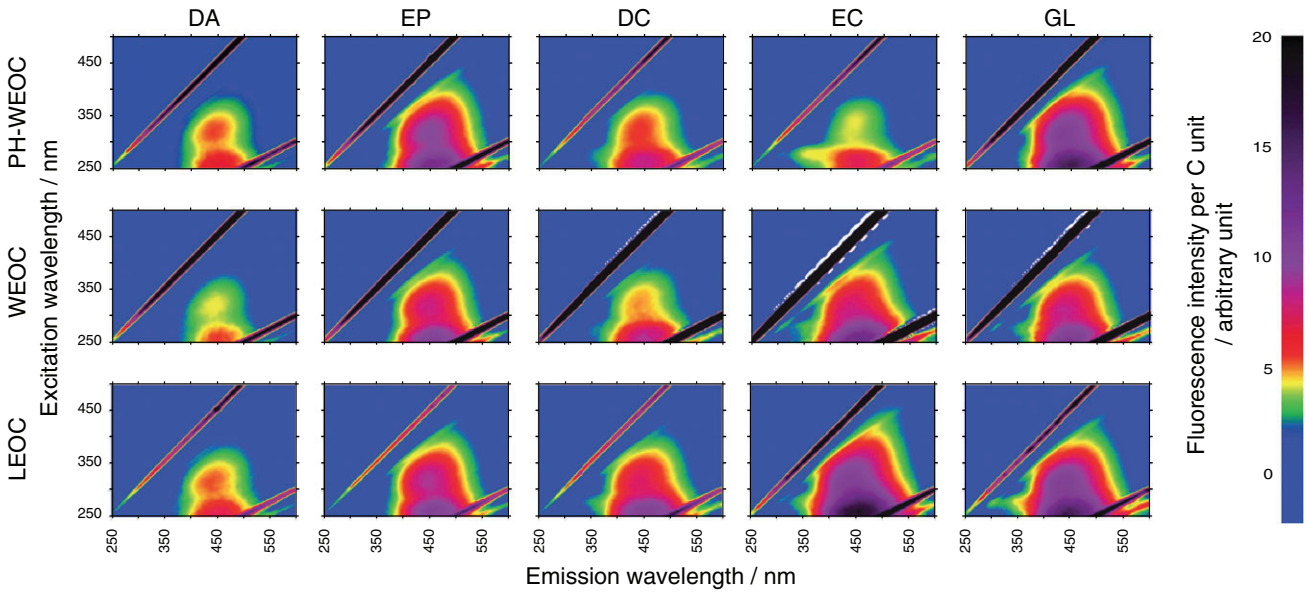

Figure 3 Fluorescence excitation-emission matrices of one replicate for each of the DOM samples extracted using pressurised hot-water-extractable organic carbon (PH-WEOC), water-extractable organic carbon (WEOC) and leaching-extractable organic carbon (LEOC) extraction procedures for the five soils. Fluorescence intensity is an arbitrary unit and all matrices were normalized in relation to their respective carbon concentrations.

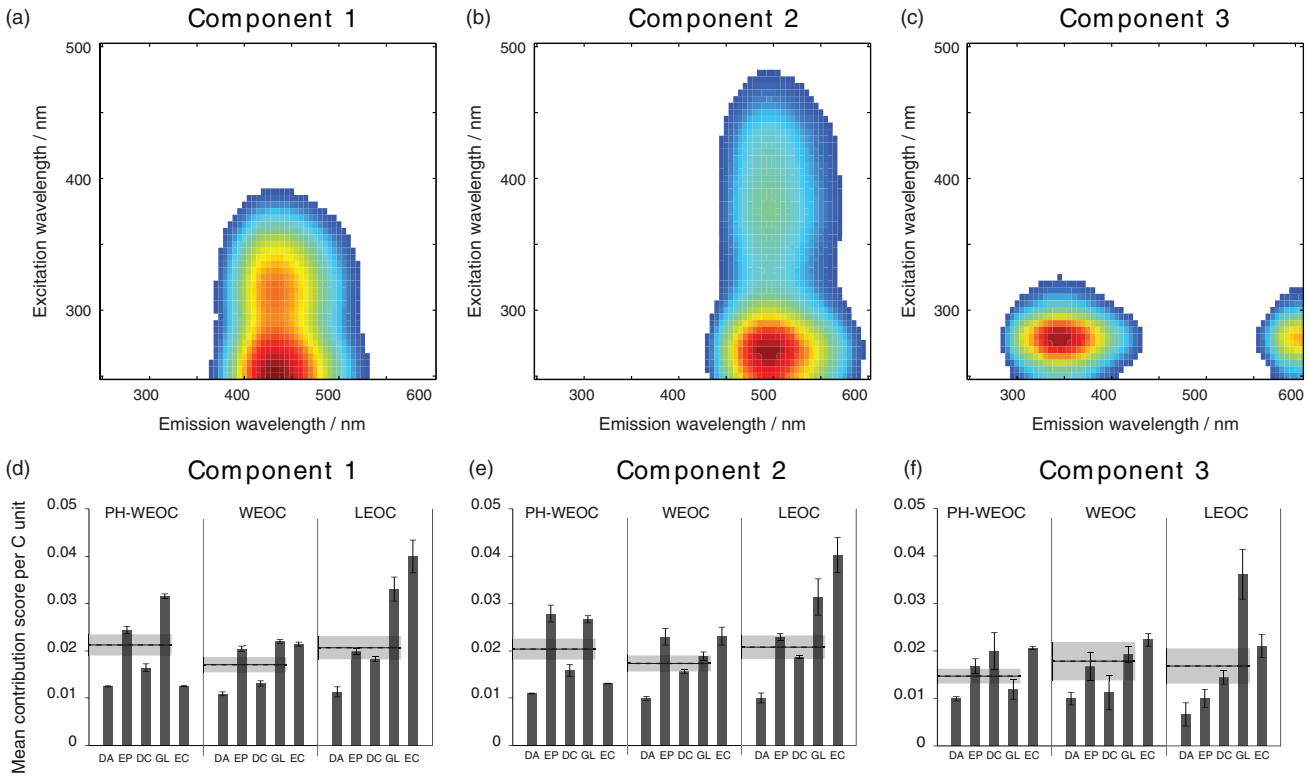

Figure 4 Fluorescence excitation-emission matrix (FEEM) representations of the three normalized PARAFAC components (a–c). Mean contribution scores of the three PARAFAC components (normalized per unit of C) for the solutions extracted from the five soils using the three methods (d–f). Component 1 is a humic-like fluorophore. Component 2 is a humic-like fluorophore with longer excitation and emission wavelengths, characteristic of a larger content of aromatic and large-molecular-weight humic materials. Component 3 is a tryptophan-like fluorophore. More details of the three components are given in the text. Error bars represent the standard error of the mean of the three replicates. For each method, the dashed line and grey band represent the mean and the standard error of the mean. The values for the EC soil were not included in the calculation of the means and the standard error of the means.

indicate why EC, which is the most alkaline soil in our study, was characterized by the larger mineralization rate constant. The WEOM extracted from soils with greater pH values may contain more easily biodegradable molecules, but the biodegradation dynamics of DOC could also be enhanced by greater pH in the incubated solution. Indeed, pH is known to control microorganism diversity and activity strongly in soils, with greater pH favouring the degradation of organic matter (Ranjard *et al.*, 2010). It is not possible here to distinguish the respective influence of each of these two different causes. The influence of pH appears to be of major importance for monitoring DOC biodegradation and we therefore recommend standardizing pH in further experiments, to assess the potential degradability of DOC from different sources in more stringently rigorous conditions.

Fluorescence analysis

Visual analysis of the fluorescence excitation emission matrices (FEEMs) showed considerable method-induced differences in fluorescence characteristics for the EC soil, while for each of the other soils the FEEMs obtained with the three methods were similar (Figure 3). However, for all of the methods, the FEEMs revealed apparent differences between the soils. All 45 samples had primary fluorescence regions around 250 nm/450 nm (λ excitation/λ emission) and 325 nm/450 nm. Another fluorescence region at approximately 275 nm/350 nm was observed, especially for EC.

This descriptive approach is a first step in explaining FEEMs. The PARAFAC modelling of FEEMs and their decomposition into individual component contributions is an adequate complementary approach, because it allows differences in fluorescence components for each sample to be quantified. The representations in FEEMs of the three normalized PARAFAC components are presented in Figure 4. In order to compare samples, contribution scores of these three components were normalized to C concentration (Figure 4).

Components 1 (Cp1) and 2 (Cp2) comprised two unresolved peaks with two excitation spectral peaks and a single emission peak. The first peak of Cp1 had a wavelength domain of about 330 nm/410–460 nm. The second peak is more intense and had a wavelength domain of about 260 nm/390–480 nm. These two fluorescence regions were respectively attributed to type C and type A humic-like compounds (Coble, 1996; Stedmon & Markager, 2005). The Cp2 covered longer wavelengths for excitation and emission (Figure 4) and had a domain of about 360–400 nm/470–510 nm for its first peak, and one of about 270 nm/450–550 nm for the more intense, second peak. The shift in longer wavelengths is characteristic of terrestrial humic-like compounds (Stedmon & Markager, 2005) and may result from a larger content of aromatic and large-molecular-weight humic materials, with a large degree of conjugation (Barančíková *et al.*, 1997). In comparison with Cp1, Cp2 is interpreted as a consequence of a more advanced maturation of organic molecules. These two humic-like fluorophores were well correlated ($r = 0.95$; $n = 45$) and will therefore be discussed together. This correlation shows that these two humic-like components are composed of molecules that are mobilized and extracted together. These two components were attributed to the results of lignin breakdown (Cory & McKnight, 2005) and have been identified in DOM from forest and agricultural streams, wastewater and seawater environments.

Table 4 Summary table for the two-way ANOVAs of contribution scores of PARAFAC components

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Log(Cp1)					
Method	2	0.284	0.142	19.46	< 0.05
Soil	3	4.147	1.382	189.43	< 0.05
Method:soil	6	0.279	0.047	6.38	< 0.05
Residuals	24	0.175	0.007	–	–
Total	35	4.885			
Log(Cp2)					
Method	2	0.172	0.086	7.13	< 0.05
Soil	3	4.802	1.601	132.48	< 0.05
Method:soil	6	0.349	0.058	4.81	< 0.05
Residuals	24	0.290	0.012	–	–
Total	35	5.613			
Log(Cp3)					
Method	2	0.088	0.044	0.25	0.78
Soil	3	3.820	1.273	7.26	< 0.05
Method:soil	6	4.457	0.743	4.23	< 0.05
Residuals	24	4.212	0.175	–	–
Total	35	12.576			

The nine observations corresponding to the EC soil were excluded from the dataset because preliminary tests revealed that these samples biased the ANOVA models.

In our samples, the results for EC extracts were not included in the ANOVAs because, once again, they biased the results, masking the main trends present in the data. We found a major effect of soil and much less pronounced effects of method and interaction on abundance in Cp1 and Cp2 (Table 4). The EP and GL extracts contain more of these humic-like fluorophores, while smaller abundances were found in DA and DC (Figure 4). These results are consistent with the soil differences observed for aromaticity and biodegradability of extracted DOC. The LSD tests revealed that the contribution scores for Cp1 and Cp2 were not statistically different for the PH-WEOC and LEOC extracts, while the WEOC method is characterised by a smaller contribution of these two components. This result revealed greater similarities in the DOM extracted by the PH-WEOC and LEOC methods, while DOM was depleted in humic-like components in the WEOC extracts.

Component 3 (Cp3) comprises two peaks with two distinct spectral emission peaks. The wavelength domain of about 275 nm/330–360 nm is attributed to peak T, a tryptophan-like fluorophore (Coble *et al.*, 1998; Stedmon & Markager, 2005), reported to be a good indicator for the size and activity of the microbial community (Cammack *et al.*, 2004; Hudson *et al.*, 2008) and to be correlated with dissolved organic N and P concentrations (Fellman *et al.*, 2009). The other peak with a maximum emission around 600 nm

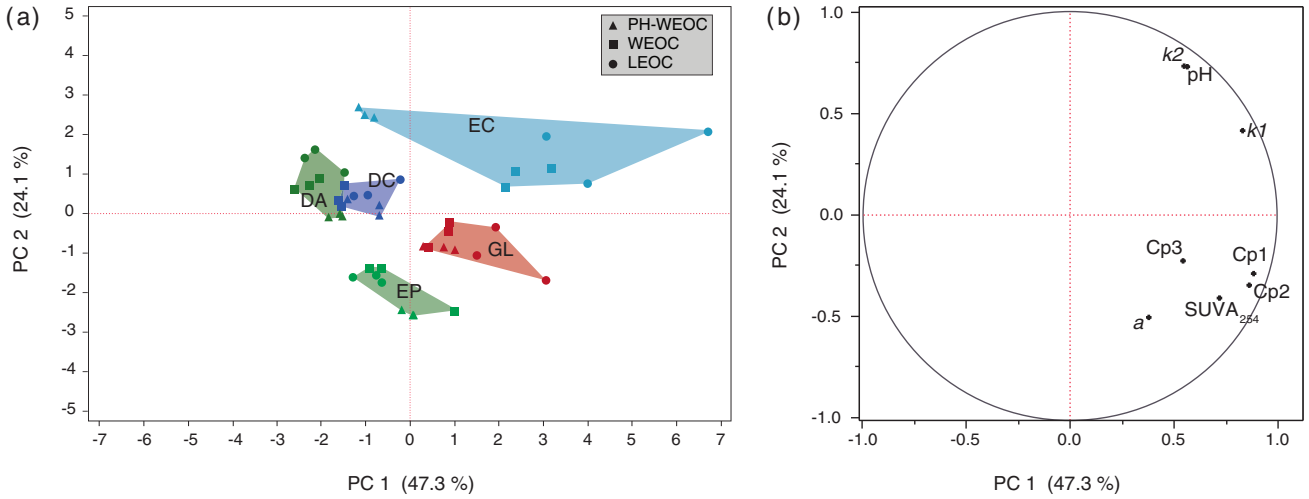

Figure 5 Score plot (a) of the principal component analysis (PCA) of the 45 DOM solutions (corresponding to the three extraction procedures, applied to the three replicates for each of the five soils) along the first (PC 1) and second (PC 2) principal components. The coloured domains correspond to the convex hulls for soils and have no statistical significance. Eight variables were investigated in the PCA (*a*, the proportion of stable DOC; *k1*, the mineralization rate constant for the labile DOC; *k2*, the mineralization rate constant for the stable DOC; pH; $SUVA_{254}$; and the three PARAFAC components (Cp 1, Cp 2 and Cp 3)). The correlation coefficients (loadings) between the original variables and the first two principal components are represented in the loading plot (b). The loadings reflect the importance of the original variables in the direction of each principal component.

is, to our knowledge, not reported in the literature, and is therefore considered as the result of noisy signals in this short excitation wavelength region (Figure 4).

For Cp3, we found a significant effect induced by the soil but not by the extraction procedure (Table 4). However, the interaction is significant, and a global view of the data shows clearly that the three extraction methods produced contrasting results when applied to the different soils (Figure 4). Therefore, this pronounced interaction makes it difficult to interpret and/or compare results concerning this tryptophan-like fluorophore in extracts obtained by any of the three methods.

To sum up the information obtained from fluorescence measurements, there are considerable differences in the fluorescence properties of the solutions extracted from the five soils (Figures 3, 4). In contrast, the effect of extraction method was less discriminant and the two most similar methods were PH-WEOC and LEOC, while WEOC was characterized by smaller proportions of humic-like fluorophores, Cp1 and Cp2. The influence of extraction is more apparent for Cp3, and we detected considerable interaction between the three methods and the soils.

Global quality of extracted DOM

The first two dimensions of the PCA space obtained from DOM qualitative characteristics are presented in Figure 5. The first principal component (PC 1), accounting for 47.3% of the variation, is mainly driven by the PARAFAC components, the $SUVA_{254}$ and *k1* (Figure 5b). For the second principal component (PC 2), explaining 24.1% of the variation, pH and *k2* contributed the most to the ordination on this second axis (Figure 5b). The PCA corroborates the discrimination between soils, as the five groups are clearly disconnected in the score plot of the PCA (Figure 5a). This pattern indicates that the soil type determines the major differences observed for WEOM quality and adequately summarizes the results reported here. The WEOM from EP and GL was enriched in humic-like and aromatic compounds and in stable DOC (*a*) whereas that from DA and DC was less aromatic, contained fewer humic-like fluorophores and had a smaller proportion of stable DOC. The PCA also demonstrates the limited impact of the extraction procedure on overall DOM quality. For soils DA, DC, EP and GL, the PCA reveals no clear patterns corresponding to a method-induced effect, whether for PH-WEOC, WEOC or LEOC, as all samples for a given soil are tightly clustered in the PCA space. The PCA therefore indicates only slight changes in the quality of the DOM obtained by the three extraction methods. The impact of method on DOM quality is much more evident for EC, with a clear separation along PC 1 for extracts obtained by each of the three extraction procedures. The alkaline pH and the presence of calcium carbonate in this soil could be a reason for its particular behaviour. The effect of calcium on WEOM was well described by Balaria (2011). Finally, PH-WEOC replicate sample locations were closer in the PCA space than those obtained with the WEOC and LEOC extraction methods. The comparisons of the pooled variance between extraction procedures reveal similar reproducibility for the WEOC and LEOC extraction methods, while the reproducibility of the extraction was significantly better for samples extracted using the PH-WEOC method.

Conclusion

This study provides evidence that WEOM quantity and quality depend primarily on soil characteristics. We found that different

extraction methods also lead to differences in the quantity and quality of WEOM. However, these differences are not overly pronounced and none of the three methods led to great differences in the chemical properties of the WEOM extracted, except for EC, an alkaline grassland soil.

The WEOC and LEOC methods give very similar results for the extraction yields (ER) and for the biodegradation characteristics. The main differences observed in WEOM obtained by these two methods were the fluorescence properties, as smaller abundances of humic-like fluorophores were found in the WEOC extracts.

In comparison with WEOC and LEOC, the extraction yields were considerably increased and reproducibility was enhanced with the PH-WEOC method. The PH-WEOC extracts were less aromatic and contained a larger proportion of stable DOC. These results showed that the increase in the extraction yields with this method corresponded to the preferential solubilisation of organic compounds with low aromaticity, which were resistant to biodegradation. The fluorescence properties of the WEOM obtained with the PH-WEOC method were very close to those observed with the LEOC method. As the PH-WEOC method did not extract WEOM with greatly different characteristics, it can therefore be used in WEOM studies.

Finally, this work confirms that caution should be exercised when comparing results obtained using different extraction methods. As results for the alkaline loamy soil sample (EC) showed, the use of different extraction methods may considerably modify the quality of the organic material extracted. Therefore further studies seem essential to develop standardized methods for the extraction of WEOM from soils. The ecological role of WEOM in soils is of primary importance and better knowledge of this role is required in order to propose a standardized extraction method.

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