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1	Article Title	**Thermally based isotopic speciation of carbon in complex matrices: a tool for environmental investigation**
2	Article Sub- Title	
3	Article Copyright - Year	**Springer-Verlag Berlin Heidelberg 2015** **(This will be the copyright line in the final PDF)**
4	Journal Name	Environmental Science and Pollution Research
5		Family Name	**Bianchini**
6		Particle	
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19		Address	Ferrara, Italy
20		e-mail	
21		Received	21 November 2014
22	Schedule	Revised	
23		Accepted	6 April 2015
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carbon pools and has been successfully applied on different environmental matrices (rocks, soils, and biological samples) with a mean C elemental and isotopic recoveries of 99.5 % (SD=1.3 %) and 0.2‰ (SD=0.2‰), respectively. The thermally based speciation (TBS) leads us to define precise isotopic end-members, which are unaffected by any chemical treatment of the sample, to be used for accurate mass balance calculation that represents a powerful tool to quantify the distinct carbon pools. The paper critically evaluates the method explaining the potentials and the current limits of the proposed analytical protocol.

Electronic supplementary material

Supplementary Fig. 1
(PDF 197 kb)

RESEARCH ARTICLE

Thermally based isotopic speciation of carbon in complex matrices: a tool for environmental investigation

Claudio Natali[1] · **Gianluca Bianchini**[1]

Abstract Elemental and isotopic analyses of carbon in environmental matrices usually highlight multiple pools of different composition and $^{13}C/^{12}C$ ($\delta^{13}C$ ‰) isotopic ratio. Interpretation necessarily needs the characterization of the diverse end-members that usually are constituted by inorganic and organic components. In this view, we developed a routine protocol based on coupling of elemental and isotopic analyses that is able to discriminate the inorganic (IC) and organic (OC) contributions to the total carbon (TC) content. The procedure is only based on thermal destabilization of the different carbon pools and has been successfully applied on different environmental matrices (rocks, soils, and biological samples) with a mean C elemental and isotopic recoveries of 99.5 % (SD= 1.3 %) and 0.2‰ (SD=0.2‰), respectively. The thermally based speciation (TBS) leads us to define precise isotopic end-members, which are unaffected by any chemical treatment of the sample, to be used for accurate mass balance calculation that represents a powerful tool to quantify the distinct carbon pools. The paper critically evaluates the method explaining the potentials and the current limits of the proposed analytical protocol.

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Introduction

The carbon elementary analysis in its distinct forms (inorganic, organic, and elemental) is of fundamental importance in the characterization of environmental matrices. Further insights on the nature of the carbon pools could be provided by isotopic analyses. Consequently, in the last few decades, thanks to the advances in technology, a widespread expansion of isotope ratio mass spectrometry (IRMS) has led to an exponential production of stable isotopes data, i.e., the $^{13}C/^{12}C$ ratio notionally expressed as $\delta^{13}C = (1000 \times [^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{standard}]/^{13}C/^{12}C_{standard})$.

In particular, for the interpretation of environmental processes, emphasis is often given to the accurate determination of key indicators such as the elemental concentration of carbon and nitrogen (and consequently the C/N ratio) of organic matter (OM) as well as its carbon isotopic composition ($\delta^{13}C$).

In this framework, uncountable applications of these environmental parameters are reported in the literature, spanning diverse scientific disciplines. For example, C/N and $\delta^{13}C$ ratios are widely used to examine OM sources, mixing, and evolution in sediments (e.g., Meyers 1997) and to identify nutrient sources and photosynthetic pathways in plants (e.g., C3 and C4 plant differentiation; Meyers 2003; Sharp 2007). In soils, these parameters provide the identifications of carbon reservoirs and dynamics in the soil–plant systems (e.g., Harris et al. 2001) and have been successfully used to identify lithogenic and pedogenic carbonates (Nordt et al. 1998), as well as the C3 vs. C4 contributions to soil organic matter (SOM), that in turn give insights on climatic conditions (Morgun et al. 2008). In agronomic studies, these tracers also

serve to evaluate trophic levels in environmental systems (Bunn et al. 1995; Pinnegar and Polunin 1999) as well as to understand mineralization processes (e.g., Freudenthal et al. 2001). Moreover, the same parameters often give insights on paleoclimate reconstruction (Leng and Marshall 2004).

However, isotopic analyses are often used for qualitative interpretation, whereas quantitative estimates are usually made with other techniques. While the TC determination is straightforward, the separation and analysis of the IC and OC fractions are more complex, time-consuming, costly, and usually require the utilization of dangerous chemicals. To achieve these results, a number of physico-chemical pre-treatments of the sample have been proposed and developed for the various environmental matrices and the associated carbon fractions, depending on the nature of the investigated samples (Walkley and Black 1934; Froelich 1980; Cachiers et al. 1989; Caughey and Barcelona 1994; Serrano et al. 2008; Meyer et al. 2013). Unfortunately, all these analytical protocols invariably require acidification of the sample to remove the IC prior to the determination of the OC fraction, either when measured directly by dry combustion (Verardo et al. 1990; Bisutti et al. 2004; Phillips et al. 2011) or by colorimetric techniques (Soon and Abboud 1991) after wet chemical oxidation (e.g., Walkley and Black 1934 for soils). This approach, employing acidification pre-treatment, although very popular, is highly contentious. Several studies investigated the effects of distinct acidification methods and acid types on different environmental matrices, indicating that the procedure variously leads to C and N losses, in turn resulting in a nonlinear, sometimes significant, deviations in C/N and δ^{13}C values (e.g., Serrano et al. 2008; Brodie et al. 2011 and reference therein; Schlacher and Connolly 2014 and reference therein).

Another way to separate IC and OC fractions can be based on thermal methods that exploit their distinctive decomposition temperature intervals. Boyle (2004) shows that, for plants and soils, most of the OM thermal destabilization occurs in the interval between 200 and 500 °C, whereas soil carbonates breakdown at temperatures between 650 and 750 °C. A more detailed study provided by Pallasser et al. (2013), based on soil thermogravimetric data obtained by dry combustion and coupled with mass spectrometry (TGA-MS), suggested a narrower temperatures range 200–430 °C for a reliable determination of soil OC to avoid contribution from inorganic sources progressively occurring at higher temperatures. This is in agreement with the temperature of 430 °C generally suggested for OM determination in soils by loss on ignition (LOI; Nelson and Sommers 1996; Chatterjee et al. 2009). Methods have been also applied to thermally resolve the presence of black carbon, the detection of which is difficult especially in complex matrices such as soils (Hsieh and Bugna 2008; Bisutti et al. 2007; Leifeld 2007). Dry combustion can also be utilized to determine IC fraction in environmental

matrices. In this case, the measurement involves samples that have to be pre-combusted to remove the OM in an O_2 stream (Rabenhorst 1988).

The abovementioned methods have been improved coupling the carbon isotope analysis, which represents a powerful tool for the discrimination of the distinct carbon pools in heterogeneous environmental materials (Lopez-Capel et al. 2006; Manning et al. 2005, 2008).

In this paper, we further explored the potential of a thermal separation method to selectively extract the different organic and inorganic fractions on the basis of their distinctive thermal stability. This thermally based speciation (TBS) is very efficient, especially if the CN-automated elemental analyzer (EA) is coupled with an IRMS; in this case, the quantitative estimate of the various carbon pools can be cross-checked and validated by C(%)-δ^{13}C(‰) mass balance calculation between the bulk values and those of OC and IC fractions.

In this contribution, we tested a TBS analytical protocol using EA-IRMS system for a precise, rapid, and economic elemental and isotopic investigation of a large variety of environmental matrices spanning from rocks to soils and biological materials. Results are discussed in order to establish the reliability of the method emphasizing the advantages with respect to alternative techniques.

Materials and methods

Investigated materials

A number of environmental matrices having different natures have been used in this study in order to test the reliability of the proposed analytical protocol for the carbon speciation on samples characterized by large organic and inorganic compositional variability. The selected sample set include two rocks (one marble from Carrara, Italy, and one carbonatic magmatic rock namely carbonatite from Jacupiranga, Brazil) as well as samples containing a variable amount of organic compounds. The latter consist of two plants (a vascular *Calluna* and a nonvascular *Sphagnum* moss previously studied by Bragazza and Iacumin 2009), one poultry litter, and one cattle manure sample. The methodology has been also tested on a set of soil samples from the Padanian Plain of Northern Italy taken in the surroundings of Argenta that were previously studied by Di Giuseppe et al. (2014). The subset selection of soils samples has been mainly made in order to cover a wide range of key chemical–physical parameters such as LOI (wt%) ranging from 9.1 (AR16A) to 14.7 (AR30A), Al_2O_3 (wt%) ranging from 12.4 (AR30A) to 20.2 (AR16A), and CaO (wt%) ranging from 1.7 (AR16A) to 13.0 (AR23B). As observed by the investigation of soils in a neighboring sector of the plain, the observed chemical–physical variations are strictly related to the grain size and Al_2O_3 and LOI tend to increase from sandy

to clayey samples (Bianchini et al. 2012). All samples have been preliminarily dried (rocks and soils at 105 °C overnight, biological samples at 40 °C for 48 h) and then powdered with an agate mortar prior to being processed.

Determination of the organic and inorganic fraction

The organic and inorganic fractions of the analyzed samples have been thermally separated by a dry-ashing method (e.g., Peacock 1992), and the percentage of organic matter (OM) has been carried out by thermo-gravimetry. Samples were loaded in ceramic crucibles previously heated at 500 °C and weighed by a high-precision balance having an accuracy up to 10 μg. The crucibles have been heated for 12 h in a muffle furnace at temperature of 450 °C, generally referred to as the temperature at which organic matter totally volatilizes (Craft et al. 1991; Ostrowska and Porębska 2012; Schrumpf et al. 2014), then placed in a silica gel-based desiccator and finally weighed to determine the gravimetric loss, representing the volatilized OM content (plus the contribution of mineral dewatering in case of clay-rich soil samples). In order to collect an appropriate amount of sample for the IC analysis, the amount of starting material processed by dry-ashing at 450 °C was variable from 5–7 mg for marble and carbonatite to 50–60 mg for plants. The measure of TC, OC, and IC has been repeated three times for all samples to carry out consistent results and to avoid outlier values. The obtained OM contents vary in the range from ca. 0 for the two rocky samples to a maximum of 96 and 98 % for the *Sphagnum* moss and *Calluna* plant, respectively. The cattle manure and poultry litter samples show intermediate OM content of 85 and 62 %, respectively. The complementary combustion residue in the form of ashed materials has been therefore defined as the IC fraction.

Alternative wet oxidation by the addition of 30 % H_2O_2 was applied to the cattle manure and poultry litter samples with the aim to remove the organic matter. The reactions took place in Pyrex® beakers which were periodically hand-shaken and left for 24 h to ensure removal of organics (Koch et al. 1997). The resulting slurries have been rinsed five times with deionized water, then filtered by 0.42 μm Whatman cellulose filter, and dried in an oven at 40 °C for 48 h. Dry weight differences highlight a removal of organic matter by wet oxidation of ca. 50 and 80 % for poultry litter and cattle manure samples, respectively.
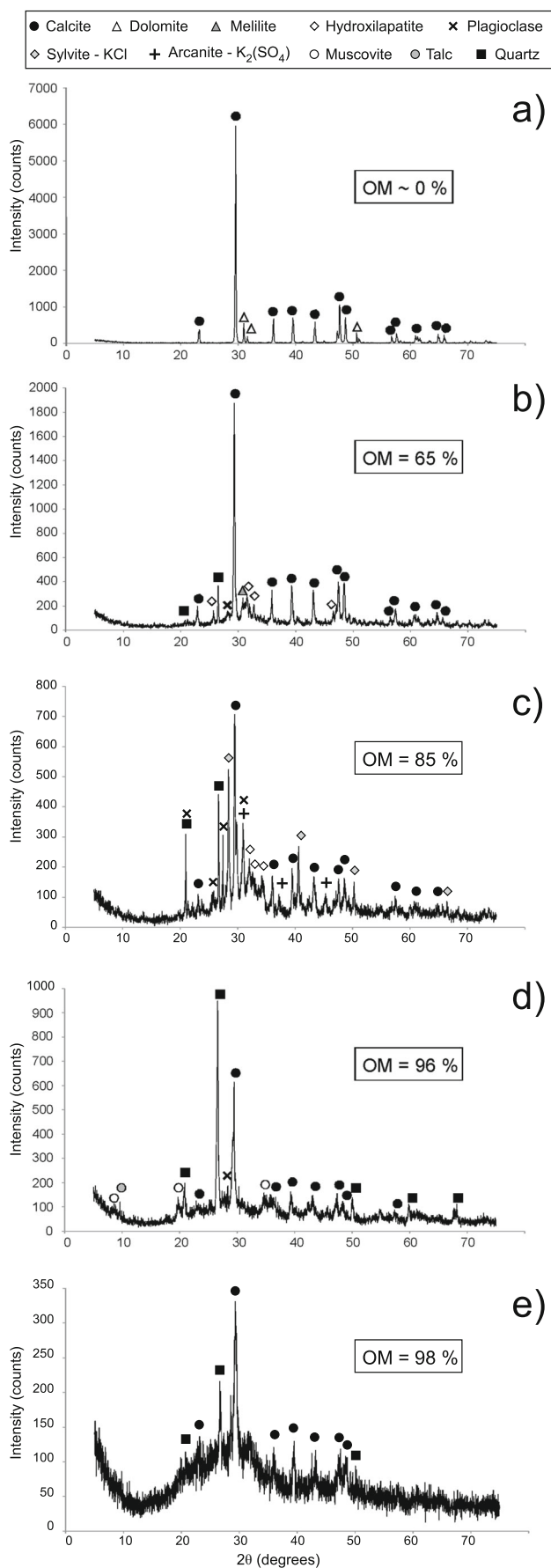
Mineralogical composition of the IC fraction

The bottom ashes resulting from the thermal treatment have been powdered in agate mill and then analyzed by X-Ray powder diffraction (XRPD) to evaluate the crystallinity degree and to characterize the mineralogical composition. Depending on the nature of the sample, a variable amount of starting material was processed at 450 °C in order to recover enough ash for the XRPD characterization (from 20 mg for rocks up to 500 mg for plants). The XRPD analysis has been carried out by a Philips PW1860/00 diffractometer using graphite-filtered CuKα radiation (1.54 Å). Diffraction patterns were collected in the 2θ angular range 5–75°, with a 5 s/step (0.02 2θ). Detection limit of distinct crystalline phases in complex matrices is notionally in the order of 3 %. Crystalline mineral phases were sequentially identified by comparison with powder diffraction files from the International Centre for Diffraction Data Powder Diffraction Files (ICDD PDF2) database.

The mineralogical composition of the IC fraction of rocky samples reveals that Carrara marble is dominated by calcite ($CaCO_3$, not shown), whereas in carbonatite the reflections at 2.89 Å suggests that dolomite—$(Ca, Mg)CO_3$—although subordinate to calcite is also present (Fig. 1a). The diffraction pattern of the combustion residue of poultry litter is characterized by a very low background coupled with sharp reflections suggesting that its IC fraction is largely free of amorphous matter and characterized by high crystallinity degree, mainly represented by calcite, hydroxylapatite ($Ca_5(PO_4)_3(OH)$), plagioclase, minor quartz (SiO_2), and minerals of the melilite group, compatible with the observed reflections in the 2.841–2.911 Å d region (Fig. 1b). Note that melilite group minerals plausibly are newly formed phases related to the combustion process, as often observed in various fired products (Bianchini et al. 2006; Maschowski et al. 2012). The IC fraction of cattle manure appears to be less crystalline and is mainly composed by calcite, hydroxylapatite, arcanite (K_2SO_4), and by minor quartz, plagioclase, and sylvite (KCl, Fig. 1c). The *Sphagnum* moss IC fraction is characterized by a low-background sharp XRPD pattern in turn indicating high crystallinity originated by the dominant presence of quartz and minor calcite, plagioclase, muscovite ($KAl_2(Si_3Al)O_{10}(OH, F)_2$)/illite, and talc (Fig. 1d). The *Calluna* IC fraction shows however lower crystallinity with only calcite and quartz as identifiable mineral phases (Fig. 1e).

Elemental and isotopic carbon speciation

The elemental and isotopic carbon composition of the different carbon pools has been carried out by the use of an Elementar Vario Micro Cube Elemental Analyzer in line with an ISOPRIME 100 Isotopic Ratio Mass Spectrometer operating in continuous flow mode. The system allows variations of the combustion module temperature up to 1050 °C; this theoretically permits extraction of different components having distinctive destabilization temperatures and to analyze the respective C(%)-δ^{13}C(‰) values. The described analytical system is configured also for the simultaneous analysis of nitrogen; while the N elemental content is always measurable and has been taken into account, the $^{15}N/^{14}N$ isotopic ratio is often

◀ **Fig. 1** X-Ray powder diffraction (XRPD) patterns showing the IC fraction mineralogical composition of **a** carbonatite rock, **b** poultry litter, **c** cattle manure, **d** *Sphagnum* moss, **e** *Calluna* plant

unreliable due to low IRMS signals and meaningless especially in the IC fraction. Powdered samples are introduced in tin capsules that are wrapped and weighed; these capsules, that allow up to 40 mg of sample, are subsequently introduced in the Vario Micro Cube autosampler to be analyzed. Flash combustion takes place in a sealed quartz tube filled with copper oxide grains (padded with corundum balls and quartz wool) which act as catalyst in excess of high purity (6 grade purity) O_2 gas. Freed gaseous species are transferred through a reduction quartz tube (at 550 °C) filled with metallic copper wires that reduce the nitrogen oxides (NO_x) to N_2. The formed analyte gases (N_2, H_2O, and CO_2), carried by dry He (5 grade purity) gas, pass through a water trap filled with Sicapent® ensuring complete removal of moisture, are sequentially separated by a temperature programmable desorption column (TPD), and quantitatively determined on a thermoconductivity detector (TCD). Sample N_2 goes directly to the interfaced IRMS for isotopic composition determination, while CO_2 is held by the TPD column, kept at room temperatures 20–25 °C. When N_2 isotopic analysis is over, CO_2 is desorbed from the TPD column by raising the temperature to 210 °C and finally reaches the IRMS compartment for the determination of carbon isotopic ratios. The detection of the distinct isotopic masses of the sample is bracketed between those of reference N_2 and CO_2 (5 grade purity) gases, which have been calibrated using a series of reference materials, in turn calibrated against IAEA international standards, such as the limestone JLs-1 (Kusaka and Nakano 2014), the peach leaves NIST SRM1547 (Dutta et al. 2006), the Carrara Marble (calibrated at the Institute of Geoscience and Georesources of the National Council of Researches of Pisa), and the synthetic sulfanilamide provided by Isoprime Ltd. Mass peaks were recalculated as isotopic ratios by the Ion Vantage software package. Reference and carrier gases of certified purity were provided by SIAD Ltd.

The elemental precision estimated by repeated standard analyses and accuracy estimated by the comparison between reference and measured values were in the order of 5 % of the absolute measured value. Uncertainties increase for contents approaching the detection limit (0.001 wt%). Carbon isotope ratios are expressed in the standard (δ) notation in per mil (‰) relative to the international Vienna Pee Dee Belemnite (V-PDB) isotope standard (Gonfiantini et al. 1995). The $\delta^{13}C$ values were characterized by an average standard deviation of ±0.1‰ defined by repeated analyses of the abovementioned standards.

The approach highlights a robust correlation between the combustion temperature and the measured carbon isotopic compositions, in relation to the distinct organic (decidedly depleted in ^{13}C) and inorganic components that are selectively

destabilized. In these tests, the sample weight varied from 1 to 20 mg for the different sample types in order to obtain significant signals (≥0.5 nA) for accurate and precise measures of the isotopic ratio. The result of these investigations has been synthetized into a rapid analytical protocol which is applicable to a wide range of environmental matrices. The protocol consists of the repeated analysis of each sample in different analytical conditions:

1. Total CO_2 stripped out after burning the sample at 950 °C, hereafter defined as TC.
2. Organic CO_2 stripped out after burning the sample at 450–480 °C, hereafter defined as OC.
3. Inorganic CO_2 stripped out after burning the abovementioned IC fraction (i.e., combustion residua after preliminary heating at 450 °C) at 950 °C.

The analyses have been repeated at least three times for each fraction in order to minimize the effect of inhomogeneity of the samples to evaluate standard deviation (SD, 1σ) and to give consistency to the method. The resulting wt% and $\delta^{13}C$ (‰) of the OC and IC fractions allow a mass balance to calculate a theoretical TC fingerprint which is compared with that directly measured ($\delta^{13}C_{TC\ Measured}$).

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$$\delta^{13}C_{TC\ Theoretical} = \delta^{13}C_{OC} \times X_{OC} + \delta^{13}C_{IC} \times X_{IC}$$

where $X_{OC} + X_{IC} = 1$ and represents the organic and inorganic fractions respectively.

The difference between theoretical and measured bulk isotopic ratios, expressed as $\Delta^{13}C$, complements the elemental carbon recovery and is used to cross-check the reliability of the method:

$$\Delta^{13}C = \delta^{13}C_{TC\ Measured} - \delta^{13}C_{TC\ Theoretical}$$

Examples and analytical details will be explained and clarified in order to validate this tool for better understanding of the carbon distribution and the related fluxes in environmental processes.

Refinement of OC and IC discrimination

In an earlier version of this analytical protocol, Natali and Bianchini (2014) studied some of the matrices reinvestigated in this contribution. In the current version, a refinement in the OC and IC discrimination leads to a great improvement in the elemental recovery as well as in the isotopic mass balance.

As concerns the IC fraction, the C (and N) elemental analysis of samples has to be corrected for the weight loss which occurred during the thermal pre-treatment; this correction (not considered in the earlier investigation) obviously has a great effect on the IC determination in totally organic matrices (weight loss close to 100 %), but is also fundamental to correctly reconstruct the bulk isotopic composition in samples containing variable amounts of organic and inorganic compounds such as soils, which can be affected by appreciable weight loss due to mineral dewatering (e.g., Boyle 2004). In addition, the effects of the combustion time (5 and 12 h) on the thermal destabilization of the OM have been also investigated; the analytical results, presented in Fig. 2, clearly demonstrate that a combustion time of 5 h (originally proposed by Natali and Bianchini 2014) is not sufficient to eliminate all the OM, as the IC fractions obtained after 12 h of thermal treatment at the same temperature (450 °C) show distinctly lower elemental C content and generally display enriched ^{13}C values, indicating a more effective removal of the OM. This is confirmed by the coupled nitrogen content (wt%) which persists at various rates, depending on the sample nature, in the 5-h experiment and is totally exhausted after the 12-h experiment.

On the other hand, a reliable quantification of the OC fraction has been evaluated through the results of the EA-IRMS dry combustion process conducted over a range of temperatures varying from 450 to 500 °C (Fig. 3). In order to fully oxidize the OM present in the sample, the procedure requires a first run combusting the introduced sample wrapped in the tin capsule, followed by a number of successive combustion runs performed on the same sample executed as "blanks." The carbon (wt%) contribution obtained by these additional combustion events progressively vanishes reiterating the runs, whereas the isotopic composition maintains the signature up to the exhaustion of OM, indicating that it has to be taken into account for a correct OC determination. In other words, the philosophy of the analytical approach to the OC quantification is to perform multiple flash combustion events on the same sample at the target temperature and to sum all the contributions of stripped CO_2 having similar "organic" isotopic signature. The number of combustion runs necessary to strip out all the organic carbon varies, depending on the nature of the sample. The specific temperature at which the combustion of the OC fraction is effective and correctly matches the elemental recovery, and the isotopic mass balance is also variable. As concerns the plants, two (or three) combustion events at temperature of 450–460 °C allow a satisfactory recovery of the organic fraction, whereas higher temperatures in the range of 470–480 °C are required for the complete organic recovery in cattle manure and poultry litter. For these latter, a distinctively less negative isotopic signature characterizes the CO_2 stripped out by the third and fourth combustion events at 460 and 480 °C. These events have not been attributed to OC and possibly mark the end of the combustion of organic matter coupled with the incipient destabilization of mineral compounds, such as oxalates, which characterize these matrices (Fig. 3).

Fig. 2 C (%) and N (%) vs δ^{13}C (‰) binary diagram showing the difference in elemental and isotopic composition of IC fractions obtained after 5 (*open symbols*) and 12 h (*filled symbols*) of thermal treatment in a muffle furnace at 450 °C. *PL* poultry litter, *CM* cattle manure, *CL Calluna* vascular plant, *SM Sphagnum* moss. *Error bars* of the isotopic analyses are smaller than the relative symbols
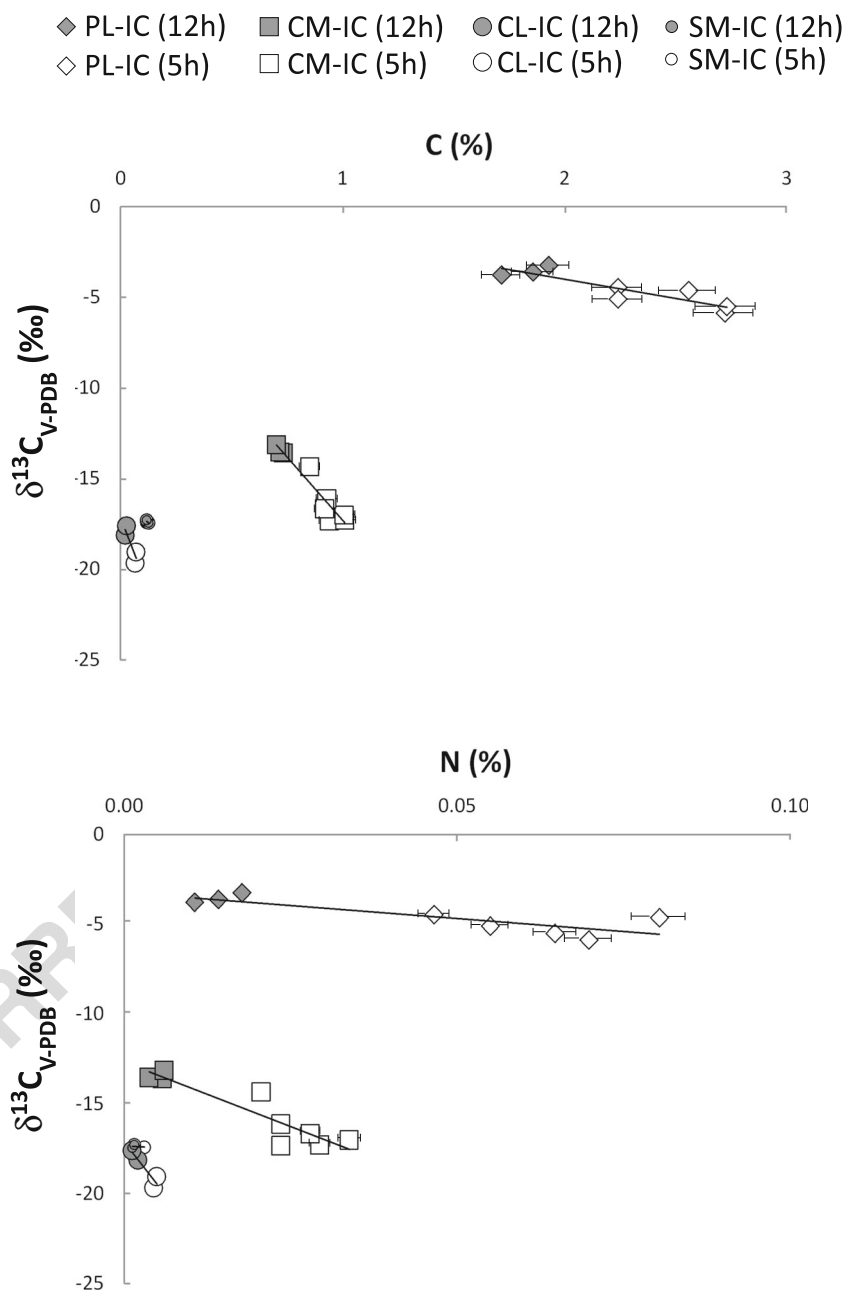

Results

The results of the elementary and isotopic carbon analyses, the associated standard deviations, and the relative recoveries are listed in Table 1. The two rock samples exhibit similar TC content (marble 12.6 %; carbonatite 12.1 %), but distinct isotopic ratios (Carrara marble: $\delta^{13}C_{TC}$=+2.1‰; carbonatite: $\delta^{13}C_{CT}$=−5.9‰) in agreement with their different genesis. The measured IC (Carrara Marble 12.6 %; carbonatite 12.2 %) is comparable with the TC content of the two rocks, as well as their respective isotopic ratios (Carrara marble $\delta^{13}C_{IC}$=2.1‰; carbonatite $\delta^{13}C_{IC}$=−6.0‰). The expected absence of organic fraction in the two rock samples is confirmed by a negligible OC content, which accounts for 0.05 % in both the Carrara Marble and the carbonatite, which is coupled with undetectable isotopic ratios due to low electronic signals. The recovery of carbon for the rocky samples is therefore excellent both for elementary (100 % for Carrara marble and 101 % for carbonatite) and isotopic composition (Carrara marble $\Delta^{13}C$=0.1‰; carbonatite $\Delta^{13}C$=0.1‰).

The two plant and the cattle manure samples show the highest TC contents (*Calluna* 54.3 %; *Sphagnum* 40.4 %; cattle manure 44.3 %) that are associated with decidedly negative $\delta^{13}C_{TC}$ values (*Calluna* $\delta^{13}C_{TC}$=−27.7‰; *Sphagnum* $\delta^{13}C_{TC}$=−26.3‰; cattle manure $\delta^{13}C_{TC}$=−24.1‰). By contrast, the poultry litter sample shows distinctly lower TC
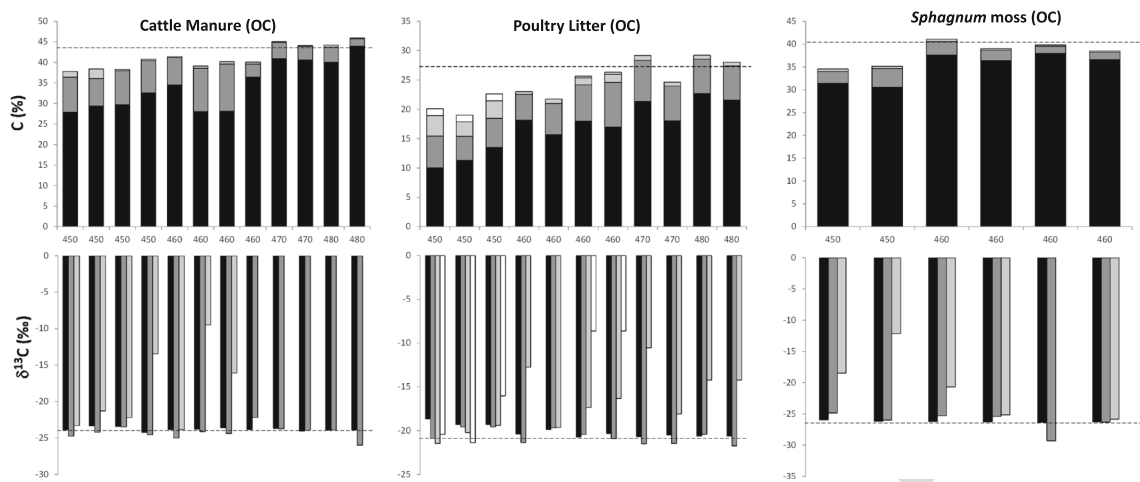
Fig. 3 Carbon elementary and isotopic composition stripped out during multiple combustion runs (*black*=first, *dark gray*=second, *light gray*=third, *white*=fourth) for the complete oxidation and quantification of the OC fraction in distinct environmental matrices. The procedure requires a first run combusting the introduced tin capsule containing the sample (*black*), followed by a number of combustion runs performed as "blanks" (from *dark gray to white*). These blanks record a C (wt%) content (28.9 %) which is coupled with a distinctly less negative isotopic value ($\delta^{13}C_{TC}$=−19.7‰). The measured IC is significantly variable in the investigated biological samples, ranging from 0.05 % (*Calluna*) to 0.16 % (*Sphagnum*) in the plants and from 0.75 % (cattle manure) to 2.1 % (poultry litter) in the animal manures. The associated isotopic compositions are similar for plants (*Calluna* $\delta^{13}C_{IC}$=−17.9‰; *Sphagnum* $\delta^{13}C_{IC}$=−17.4‰) and cattle manure ($\delta^{13}C_{IC}$=−13.7‰), whereas poultry litter IC fraction is characterized by a distinctively less negative isotopic ratio ($\delta^{13}C_{IC}$=−3.6‰). The measured OC content in plants accounts for 54.1 and 39.2 % in *Calluna* and *Sphagnum,* respectively, whereas it measures 44.0 % in the cattle manure and 27.1 % in poultry litter. In all the biological samples, the OC fraction shows an isotopic composition comparable to that associated to TC fraction (*Calluna* $\delta^{13}C_{OC}$=−27.6‰; *Sphagnum* $\delta^{13}C_{OC}$=−26.3‰; cattle manure $\delta^{13}C_{OC}$=−23.9‰; poultry litter $\delta^{13}C_{OC}$=−20.8‰). The elementary carbon recovery is very good to excellent for all the investigated samples, varying from 98 to 100 % in plants and 101 % in cattle manure and poultry litter samples. The isotopic difference ($\Delta^{13}C$) between the measured bulk composition and that obtained by mass balance calculation is very low (−0.4‰ for cattle manure, −0.2‰ in *Calluna*) to negligible (−0.1 and 0.0‰ for *Sphagnum* and poultry litter, respectively).

The soils selected from the sample set studied by Di Giuseppe et al. (2014) are characterized by a TC content varying in the range 1.3 (AR16A)–3.6 % (AR30A) and by a remarkable variation in the associated bulk isotopic composition ($\delta^{13}C_{CT}$ −5.0‰ in AR19B to −21.9‰ in AR16A). The measured IC content ranges from 0.14 % (AR16A) to 2.4 % (AR6B) and displays $\delta^{13}C_{IC}$ varying from −1.5‰ (AR30A)

contribution progressively vanishing at increasing run numbers, whereas the isotopic composition maintains the signature up to the exhaustion of OM. Experiments have been conducted at different combustion temperatures (450, 460, 470 and 480 °C) to investigate the OM oxidation dynamics in samples characterized by different nature. *Dashed lines* represent the expected elemental and isotopic values for OC considering the measured TC and IC fractions

to −10.4‰ (AR16A), whereas the OC content is in the range 0.3 % (AR19B) to 1.2 % (AR30A) and is associated with carbon isotopic ratios varying from −22.0‰ (AR34B) to −25.5‰ (AR30A). Recoveries vary from 97 % (AR16A) to 100 % (AR30B) for elemental carbon, whereas $\Delta^{13}C$ ranges from 0.0 (AR16A) to −0.6‰ (AR19B). The measured elemental and isotopic data for the selected soil samples are presented in Table 2, which also include the theoretical isotopic composition obtained by mass balance calculation ($\delta^{13}C_{CT\ Theoretical}$) for a direct comparison with the measured bulk $\delta^{13}C_{CT.}$

Factors controlling the $\Delta^{13}C$ value

The $\Delta^{13}C$ value represents a proxy to check the validity of the proposed speciation technique because it compares the sum of the distinct carbon fractions separated by this method with the bulk isotope value independently measured. In our view, the technique gives insights on the nature and origin of the mineralogical components that are detected in the burnt residua of the investigated biological matrices. As demonstrated by XRPD results, the principal carbon repository of these burnt residua is represented by calcium carbonate, which is a well-known constituent mineral phase after the combustion process of biomasses (Kuhlbusch et al. 1996; Brochier and Thinon 2003; Regev et al. 2011). A percentage of the carbonate present in the ashed material can have a secondary origin (i.e., it was not contained in the original, pre-combustion material) and its formation can be ascribed both (1) to the mineralization of CO_2 freed by the thermal decomposition of organic compounds and (2) by the thermal decomposition of biogenic oxalates. In the first case, the amount of secondary carbonates

Q6 **Table 1** Elemental and isotopic composition of distinct carbon fractions (standard deviation in parentheses) measured on different environmental matrices by thermal speciation using TBS EA-IRMS (see text for further details)

Sample	C (%)	$\delta^{13}C_{V-PDB}$ (‰)	C recovery (%)	$\Delta^{13}C$ (‰)
Marble				
TC	12.7 (0.69)	2.1 (0.2)		
OC	0.05 (0.03)	nd	0.42	
IC	12.6 (0.81)	2.1 (0.3)	99.50	
			100	0.1
Carbonatite				
TC	12.1 (0.50)	−5.9 (0.1)		
OC	0.05 (0.01)	nd	0.43	
IC	12.2 (0.67)	−6.0 (0.1)	100.30	
			101	0.1
Cattle manure				
TC	44.3 (0.77)	−24.1 (0.1)		
OC	44.0 (1.08)	−23.9 (0.2)	99.35	
IC	0.75 (0.11)	−13.7 (0.3)	1.69	
			101	−0.4
Poultry litter				
TC	28.9 (0.52)	−19.6 (0.2)		
OC	27.1 (1.24)	−20.7 (0.4)	94.00	
IC	2.00 (0.31)	−3.6 (0.3)	6.91	
			101	0.0
C3 plant (*Callunia*)				
TC	54.3 (2.51)	−27.7 (0.2)		
OC	54.1 (2.3)	−27.6 (0.4)	99.70	
IC	0.05 (0.4)	−17.9 (0.4)	0.09	
			100	−0.2
C3 plant (*Sphagnum*)				
TC	40.4 (1.13)	-26.3 (0.1)		
OC	39.2 (0.8)	-26.3 (0.1)	97.14	
IC	0.16 (0.11)	-17.4 (0.1)	0.39	
			98	−0.1

TC total carbon, *OC* organic carbon fraction, *IC* inorganic carbon fraction, $\Delta^{13}C$ difference between measured and theoretical isotopic bulk compositions

formed by combustion is specific for each biological matrices because it is strictly dependent on the presence of the alkaline elements (mainly Ca, Mg, Na, and K) which can bond to and mineralize CO_2, whereas in the second case it depends on the amount of oxalate originally present in the sample and by the combustion temperature. The occurrence of calcium oxalates in plants is well-known, but their identification is not always straightforward due to the variability of their morphology and hydration state (Franceschi and Horner 1980; Franceschi and Nakata 2005). Their isotopic composition is highly variable, generally reflecting that of the organic materials from which they derive but shifted toward more positive values (ca. 5–7‰ in woods, Cailleau et al. 2011; 6.5‰ in lichens, Beazley et al. 2002). The thermal transformation of the calcium oxalate crystals (whewellite, $CaC_2O_4 \times H_2O$, weddellite, $CaC_2O_4 \times 2H_2O$) to calcite occurs in burned plant materials at temperature ranging 430–500 °C by the loss of CO following dehydration at 100–200 °C (Kloprogge et al. 2004). According to experimental evidence provided by Manning et al. (2008), this phase transition is accompanied by loss of depleted ^{13}C CO gases coupled with a ^{13}C enrichment of the resulting calcite, with isotopic fractionation in the order of 4–6‰. In this light, the presence of oxalate could be a factor influencing the extent of the $\Delta^{13}C$ value.

Elevated $\Delta^{13}C$ values are also expected in matrices containing significant amounts of recalcitrant C-bearing phases such as mineralized organic matter and/or charred materials that are sometimes recorded in soils (Gelinas et al. 2001; Leifeld 2007).

Discussion

The elementary TC (and IC) content of the investigated rocks is in agreement with stoichiometric C content (wt %) in the $CaCO_3$ molecule which accounts for ca. 12 wt%. The $\delta^{13}C_{TC}$ values of the Carrara marble (+2.1‰) is in the typical carbonate range (Hudson 1977) and very close to the values listed for

Table 2 Elemental and isotopic composition of distinct carbon fractions measured on agricultural soils by TBS EA-IRMS technique. Abbreviations as in Table 1 and in the text

Sample	Measured						C recovery (%)	Theoretical	
	TC (%)	IC (%)	OC (%)	$\delta^{13}C_{TC\ V-PDB}$ (‰)	$\delta^{13}C_{IC\ V-PDB}$ (‰)	$\delta^{13}C_{OC\ V-PDB}$ (‰)		$\delta^{13}C_{TC}$ (‰)	$\Delta^{13}C$ (‰)
AR6B	2.88	2.39	0.47	−5.2	−2.0	−23.3	99	−5.5	−0.3
AR19B	2.12	1.77	0.34	−5.0	−2.3	−22.3	99	−5.6	−0.6
AR30B	2.74	2.33	0.4	−4.4	−1.8	−21.9	100	−4.7	−0.3
AR23B	2.94	2.46	0.45	−4.4	−1.8	−21.6	99	−4.9	−0.5
AR16A	1.31	0.15	1.13	−21.9	−10.4	−23.4	97	−21.9	0.0
AR30A	3.56	2.24	1.3	−10.1	−1.5	−25.5	99	−10.3	−0.3

Carrara marble by Cortecci et al. (2003) and for "Carrara marble-C1" reference material certified by IAEA (+2.42‰). The $\delta^{13}C_{TC}$ of carbonatite sample (−5.9‰) exhibits a composition typical of Earth's mantle ($\delta^{13}C$ to approximately −5‰; Deines 2002) and approaches the literature values reported for the Jacupiranga carbonatite rocks (ca. −6.4‰; Santos and Clayton 1995). The difference between $\delta^{13}C_{TC}$ and $\delta^{13}C_{IC}$ for these rocks ($\Delta^{13}C=0.05‰$) is well below the analytical error ($\delta^{13}C\pm0.1‰$). As expected, the elementary OC fraction in the investigated rocky samples is almost undetectable (0.05 %), and the interpretation of which is not so straightforward due to the low signal on the mass spectrometer leading to an undeterminable associated isotopic ratio. Although insignificant from the methodological point of view, the systematic measure of a small OC fraction in these rocks should likely not be assigned to organic matter present in the sample, but more probably to the incipient decomposition of dolomite (Gatta et al. 2014) or of metastable mineral phases (Carmody 2012). Notably, the presence of dolomite in the studied rocks is documented in the literature (Merlino and Orlandi 2001; Alva-Valdivia et al. 2009). Alternative hypotheses explaining the low-temperature CO_2 release from the Carrara marble could be ascribed to "impurities" in the protolith, i.e., sporadic presence of organic derived components in the precursor sediment (Sreenivas et al. 2001), or to the presence of fluid inclusions that can release non-condensable gases (Sisson and Hollister 1990). The XRPD analyses confirm the significant presence of dolomite in the IC fraction of carbonatite sample, while in Carrara marble this phase is probably under the detection limit of the analytical technique.

As concerns the investigated plants, the measured OC content corresponds to a fraction of TC ranging from 1.00 in *Calluna* to 0.97 in *Sphagnum* and is associated with very negative isotopic ratios ($\delta^{13}C_{OC}$ to approximately −27‰), reflecting the typical ^{13}C depletion occurring in tissues of plants with C3 photosynthetic pathway (Sharp 2007). As expected, the recorded elemental OC in the two plants is in agreement with the respective OM determination (98 % in *Calluna* and 96 % in *Sphagnum*). The EA analysis of the plant IC fractions confirms that they bear a complementary carbon content (0.05 % in *Calluna* and 0.16 % in *Sphagnum*). In plants, the IC/TC ratio varies from 0.09 (*Calluna*) to 0.39 (*Sphagnum*) and is coupled with a $\delta^{13}C_{IC}$ decidedly less negative than that of the relative OC (and TC), excluding a primary origin for the calcite recorded by the XRPD spectra of their ashed material (Fig. 1d, e). Moreover, the isotopic difference between the bulk $\delta^{13}C$ (or the $\delta^{13}C_{OC}$) and the $\delta^{13}C_{IC}$ accounts for ca. 9‰ in *Calluna* and 10‰ in *Sphagnum* samples. Such isotopic fractionation is compatible with that of secondary calcite produced by thermal decomposition of biogenic oxalate (Cailleau et al. 2011; Manning et al. 2008). The resulting $\Delta^{13}C$ (−0.2 and −0.1‰ for *Calluna* and *Sphagnum*, respectively) is very low.

The cattle manure sample shows an OC/TC ratio (0.99) similar to that recorded in plants, whereas the OC isotopic ratio is slightly less negative ($\delta^{13}C_{OC}=−23.9‰$) in comparison with those of investigated plant materials. The value of $\delta^{13}C_{OC}$ conforms however with that of digestion products of animals having a dietary regime mainly constituted by C3 vegetation (Deniro and Epstein 1978). The complementary IC fraction of cattle manure is slightly higher (IC/TC=0.17) than that of plants and is characterized by a more complex mineralogical composition (Fig. 1c). The associated $\delta^{13}C_{IC}$ (−13.7‰) is ca. 10‰ less negative than bulk $\delta^{13}C_{TC}$ (and $\delta^{13}C_{OC}$) recording a fractionation similar to that observed in burnt plant samples. In general, the elemental and isotopic carbon speciation reflects the composition of cattle manure which is mainly constituted by organic feedstock residua and possibly by a mineral fraction (including calcium oxalates; Shahack-Gross 2011). Although very good, the resulting $\Delta^{13}C$ is higher (−0.4‰) than for plants, and this is probably due to a higher proportion of oxalates constituting the original mineral fraction, as detected by the XRPD analysis of H_2O_2-treated sample (Supplementary Fig. 1a), which totally transformed into carbonates during the thermal production of the IC fraction, leading to slight isotopic enrichment of this latter.

The poultry litter sample shows the lowest OC fraction (OC/TC=0.94) and OM determination (67 %) among the investigated biological matrices. It is the only investigated samples showing an OC isotopic composition distinctly more negative than that of the bulk ($\delta^{13}C_{TC}$-$\delta^{13}C_{OC}=1.2‰$). As expected, it bears the highest IC fraction (IC/TC ca. 0.07) displaying the most ^{13}C-enriched isotopic signature ($\delta^{13}C_{IC}=−3.6‰$), which approaches that of typical of primary carbonates. This is confirmed by the comparison of XRPD analysis of the IC fraction with that of H_2O_2-treated subsample, showing that calcite is predominant with respect to oxalate in the original poultry litter sample (Supplementary Fig. 1b), leading to $\Delta^{13}C$ approaching 0.0‰.

Summarizing, the carbon isotopic composition of the IC fraction provides useful insights to understand and quantify the contribution of primary and secondary carbonates. The elemental and isotopic carbon recovery efficiency is very high for all the investigated samples and the lowest elemental recovery (ca. 98 %) has been observed for *Sphagnum* moss sample.

In our view, the method is ideal to study complex matrices in which the presence of distinct carbon forms is ubiquitous. For example, the application to soils from the Padanian plain (Northern Italy; Bianchini et al. 2012) allows the precise determination of their TC, IC, and OC which implements previous investigations in terms of geochemistry, geomorphological setting, and sampling depth (Di Giuseppe et al. 2014). The IC tends to prevail in the soils developed from coarse sediments deposited in high energy hydrodynamic conditions. On the contrary, OC becomes prevalent in soils developed from

fine-grained sediments from low-energy depositional environments. The isotopic composition of IC recorded in the majority of the investigated soils is very similar (average $\delta^{13}C_{IC}=$ −1.9‰, SD=0.3‰) confirming both the lithogenic nature of the measured fraction and its stability through the geological conditions. However, an exception is represented by the sample AR16A which shows $\delta^{13}C_{IC}=$−10.1‰, plausibly reflecting the presence of pedogenic carbonates in its mineralogical fraction. In fact, the recorded $\delta^{13}C_{IC}$ for this sample is characterized by an isotopic shift of ca. +15‰ with respect to the associated soil organic matter ($\delta^{13}C_{OC}=$−23.4‰) conforming to the values suggested by Nordt et al. (1998) for pedogenic carbonates.

In general, the $\delta^{13}C_{OC}$ values are significantly more negative than $\delta^{13}C_{IC}$ clearly deriving from the thermal destabilization of the SOM. In particular, in all the investigated samples, the isotopic composition of the TOC (average $\delta^{13}C_{OC}=$−23.4 ‰, SD=1.4‰) indicates that the SOM is mainly derived from C3 photosynthetic pathway vegetation. The recorded isotopic variability of SOM in the selected samples ($\delta^{13}C_{OC}$ from −21.6 to −25.5‰) possibly reflects isotopic fractionation originated by organic matter decomposition, leading to a production of depleted ^{13}C soil respiration gases in parallel with a ^{13}C enrichment of the residual decomposition products (Winn et al. 2006). The carbon elemental recovery is usually better than 3 % and appears not to be related to the $\Delta^{13}C$, which varies from 0.0 to −0.5‰. Note that the smallest $\Delta^{13}C$ typically denotes the samples characterized by significant SOM fraction, whereas the worst isotopic mass balance results characterize the samples having the less negative $\delta^{13}C_{OC}$ values. This means that a carbon fraction with distinctly negative isotopic signature has not been measured during the three steps of the analytical procedure. Taking into consideration the experimental evidence provided by Leifeld (2007) on the thermal stability of black carbon, we suggest that the inferred unmeasured fraction is plausibly represented by charred material known to be recalcitrant to combustion processes at temperature <480 °C and/or by elemental carbon plausibly related to incipient SOM mineralization.

Conclusions

Recent analytical improvements have boosted the thermal analysis as a promising approach to investigate carbon pools in multiphase environmental matrices (Bisutti et al. 2007; Vuong et al. 2013). Thermally based carbon speciation is favored with respect to other methods due to limited sample manipulation, rapidity, and cost-effectiveness. The research presented in this work demonstrates that isotopic analysis represents an additionally powerful tool that can be used to complement the TBS method. The mass balance calculations outlined above consider both elemental and isotopic data that

offer an accurate cross-check for the verification of the carbon pools delineation. The proposed method maximizes the potential of an EA-IRMS system through variation of the combustion temperature in order to match the specific destabilization conditions of the targeted carbon compounds. In particular, the OC analysis varies in temperature from 460 to 470 °C, and multiple runs are always necessary to obtain a complete oxidation of the OM. Precise operative details are required on a case-by-case basis. The establishment of such operating details needs to be determined through monitoring of obtained isotopic data. This delineation will outline tailor-made conditions (temperature and iterations necessary to exhaust OM) for specific matrices. In particular, C (wt%)–$\delta^{13}C$ (‰) mass balance can be useful for deciphering the presence of phases characterized by overlapped destabilization temperatures that are difficult to be resolved with notional TBS method. The philosophy of the method could be refined in order to decipher distinct C forms/types with possible applications in the characterization of contaminated soils and sediments.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. (Country / City) has been provided, please check if it is correct.
Q2. Caughney and Barcelona, 1994 has been changed to Caughey and Barcelona 1994 as per the reference list. Please check if okay.
Q3. Leifield, 2007 has been changed to Leifeld 2007 as per the reference list. Please check if okay.
Q4. Kock et al., 1997 has been changed to Koch et al. 1997 as per the reference list. Please check if okay.
Q5. Please check equations if captured and presented correctly.
Q6. Please check Tables if captured and presented correctly.