Isolating organic carbon fractions with varying turnover rates in temperate agricultural soils – A comprehensive method comparison

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Abstract

Fractionation of soil organic carbon (SOC) is crucial for mechanistic understanding and modeling of soil organic matter decomposition and stabilization processes. It is often aimed at separating the bulk SOC into fractions with varying turnover rates, but a comprehensive comparison of methods to achieve this is lacking. In this study, a total of 20 different SOC fractionation methods were tested by participating laboratories for their suitability to isolate fractions with varying turnover rates, using agricultural soils from three experimental sites with vegetation from C3 to C4 22-36 years ago. Enrichment of C4-derived carbon was traced and used as a proxy for turnover rates in the fractions. Methods that apply a combination of physical (density, size) and chemical (oxidation, extraction) fractionation were identified as most effective in separating SOC into fractions with distinct turnover rates. Coarse light SOC separated by density fractionation was the most C4-carbon enriched fraction, while oxidation-resistant SOC left after extraction with NaOCl was the least C4-carbon enriched fraction. Surprisingly, even after 36 years of C4 crop cultivation in a temperate climate, no method was able to isolate a fraction with more than 76% turnover, which challenges the link to the most active plant-derived carbon pools in models. Particles with density >2.8 g cm^{-3} showed similar C4-carbon enrichment as oxidation-resistant SOC, indicating the importance of sesquioxides for SOC stabilization. The importance of clay and silt-sized particles (<50 µm) for SOC stabilization was also confirmed. Particle size fractionation significantly outperformed

aggregate size fractionation, due to the fact that larger aggregates contain smaller aggregates and organic matter particles of various sizes with different turnover rates. An evaluation scheme comprising different criteria was used to identify the most suitable methods for isolating fractions with distinct turnover rates, and potential benefits and trade-offs associated with a specific choice. Our findings can be of great help to select the appropriate method(s) for fractionation of agricultural soils.

1 Introduction

Fractionation of soils to gain a better understanding of element cycling within a ‘black box’ system has a long history. For soil organic carbon (SOC), the techniques applied have evolved according to the current understanding of carbon (C) stabilization and turnover in soils. The traditional view of SOC stabilization was that dead plant material becomes ‘humified’, a process which involves secondary synthesis of ‘humic substances’ that become chemically stabilized against microbial decay (Stevenson, 1994; Burdon, 2001). In this approach, SOC is characterized using alkaline extraction, isolating ‘humic acid’, ‘fulvic acid’ and ‘humin’. The first report of such a procedure dates back to 1786 (Achard, 1786). However, it has been pointed out that this concept may not be completely applicable to C turnover processes in soils, since: i) there is no evidence that synthesized ‘humic substances’ actually exist under natural conditions (Lehmann and Kleber, 2015) and ii) there is evidence that the availability of a substrate for degraders is more important for their persistence in the soil than its chemical recalcitrance (Kögel-Knabner,

2002; Denef et al., 2009; Dungait et al., 2012). Nonetheless, different views on the fate of organic matter in soils still persist to date (Lützow et al., 2006; Nebbioso and Piccolo, 2011; Lehmann and Kleber, 2015), owing to: i) the complex nature of SOC and soil organic matter in general, ii) the diversity of potential stabilization mechanisms, and iii) the limited ability to study organic matter molecules in the soil at sufficient temporal and spatial resolution.

The diversity of mechanistic theories regarding turnover, stabilization, and formation of SOC and different goals in measuring SOC and its pools are reflected in the wide range of fractionation methods currently applied (von Lützow et al., 2007). While some methods are designed purely to assess turnover, others might reveal mechanistic details of how SOC is formed and interacts with the soil matrix. Each method has its own rationale and has a more or less extensive community of users and supporters. The majority of the more recently developed SOC fractionation methods use physical fractionation approaches, such as separation of particles by density and/or size, with or without previous dispersion to break aggregate structures (Golchin et al., 1994b; Six et al., 2002a; Sollins et al., 2006). This approach emphasizes the importance of the fundamental interactions between organic and inorganic soil components in the turnover of organic matter (Christensen, 2001). Physical protection by aggregates and by organo-mineral complexes (especially in the silt and clay-sized fractions) is acknowledged to be crucial for SOC stabilization (Six et al., 2002b; Eusterhues et al., 2003; Kaiser and Guggenberger, 2003). In contrast, chemical extractions are applied for advanced chemical characterization of SOC, based on the concept that chemical quality and inherent recalcitrance are of major importance for SOC stabilization (Olk and Gregorich, 2006). Extraction with water is applied to isolate dissolved organic carbon (DOC), a highly mobile C fraction (Michalzik et al., 2003). Chemical oxidation is performed to mimic strong enzymatic decay (von Lützow et al., 2007). Since both approaches, physical and chemical fractionation, may have their shortcomings regarding the isolation of meaningful, distinct functional pools, combined approaches of chemical

and physical fractionation have emerged (Plante et al., 2006; Zimmermann et al., 2007b). In these, size or density separation is often used to isolate mineral-associated SOC, which is then chemically treated to separate an oxidation-resistant fraction. Frequently used oxidation agents are hydrogen peroxide (H_2O_2) (Eusterhues et al., 2005), sodium hypochloride (NaOCl) (Kaiser and Guggenberger, 2003), and sodium persulfate ($Na_2S_2O_8$) (Eusterhues et al., 2005; Helfrich et al., 2007). However, critics point out that chemical and biological oxidation are not the same and are thus driven by different SOC properties (Leifeld and von Lützow, 2014; Lutfalla et al., 2014). Similar criticisms have been made of thermal oxidation methods, which are believed to derive fractions differing in biological stability via stepwise thermal oxidation (Helfrich et al., 2010; Schiedung et al., 2017). An alternative to chemical treatment of size fractions is the use of spectral methods (e.g., nuclear magnetic resonance) to estimate the resistant carbon within size fractions (Guggenberger et al., 1994; Six et al., 2001; Baldock et al., 2013). However, this method does not allow for separation and isolation of the final fraction components.

The existing SOC fractionation methods have been developed for different ecosystems and soils and to answer different research questions. However, they are frequently used for one single purpose, which is to isolate SOC pools that are as homogeneous and distinct in their turnover rates as possible (Trumbore and Zheng, 2016). This is challenging, since SOC comprises a wide range of different components with ages ranging from hours to millennia (Trumbore et al., 1989; Paul et al., 1997). Fraction-derived C pools are used to develop, initialize, and validate mechanistic models of SOC turnover (Segoli et al., 2013), and to characterize SOC regarding its formation and stability (Baldock et al., 2013; Cotrufo et al., 2015), in undisturbed conditions or following environmental perturbation. Several studies have been able to link empirically isolated fractions to the theoretical, kinetically delineated components of SOC (i.e., pools) of the RothC model (Balesdent, 1996; Skjemstad et al., 2004; Zimmermann et al., 2007b). However, an empirical link, i.e. comparable distribution of carbon in fractions and pools, does not necessarily mean a functional link,

i.e. that isolated fractions or fraction combinations and model pools have a similar turnover or respond to changes in a similar way (Poeplau and Don, 2013b). To evaluate the mean residence time of a certain fraction or to have a proxy for its turnover rate, either ^{14}C measurements (Marzaioli et al., 2010) or environmental changes (land use, land management, soil temperature) ideally creating a shift in ^{13}C abundance or other biomarker are necessary (Del Galdo et al., 2003; Dondini et al., 2009). However, such an evaluation has not previously been broadly applied across commonly used fractionation methods.

The diversity and large number of fractionation methods hamper quantitative comparisons between studies and model initialization across studied soils. Ideally, all scientists with a common goal would apply only one optimized and standardized fractionation method to all soils. This is believed to be difficult, because soils differ substantially in their prevailing SOC stabilization mechanisms and many other properties. Hence, it might be unlikely that one method is best suited for all soils. However, to date it is hard to judge, whether the huge variability of methods applied is justified, or if certain method types or fractionation steps would generally be more effective than others in isolating fractions with distinct properties.

The aim of this study was to set up a comprehensive comparative fractionation experiment with as many different fractionation methods as possible. Those methods were then aimed at being compared regarding their ability to isolate C fractions with different turnover rates in temperate agricultural soils. In addition, a set of performance metrics to support method comparisons and decisions on choice of a specific method was set up.

2 Materials and Methods

2.1 Concept and soils

The change in ^{13}C abundance induced by a shift from C_3 to C_4 vegetation was used to derive an indicator for the turnover rate or persistence of C in

each isolated fraction. The separation into the two sources of vegetation is based on the fact that C3 photosynthesis (Calvin cycle) has a higher discrimination against ^{13}C than C4 photosynthesis (Hatch-Slack pathway) (Farquhar et al., 1989). Therefore, the $\delta^{13}C$ values from plants with C_3 photosynthesis are typically about -28‰, while those from plants with C4 photosynthesis scatter around -12‰. The space-for-time approach was used here, i.e., sampling a C3 reference soil and an adjacent soil with a C3-C4 vegetation change at a known point in time. The incorporation of new C4-derived C can then be determined for each fraction using the following two-pool mixing model (Balesdent et al., 1987):

$$f_{C4_{fraction_i}} = \frac{\delta^{13}C_{fraction_i(C4soil)} - \delta^{13}C_{fraction_i(C3soil)}}{\delta^{13}C_{C4plant} - \delta^{13}C_{fraction_i(C3soil)}},$$

(Eq. 1)

where f_{C4} is the proportion of C4-derived C in the SOC $fraction_i$ of interest in the C4-vegetated soil; and the $\delta^{13}C$ values of $fraction_i$ (C4 soil), $fraction_i$ (C3 soil), and C4 plant refer to the $\delta^{13}C$ values of the SOC fraction of interest in the C4 vegetated soil, the same SOC fraction in the C3 reference soil, and the $\delta_{13}C$ value of the grown C4 plant, respectively. Here, the latter was set to -12‰ for all three soils studied (Menichetti et al., 2013). The bulk soil f_{C4} was calculated with the same equation. Based on the assumption that the system is at steady-state, a fraction with a relatively high enrichment of C4-derived C (f_{C4}~1) would thus be interpreted as fast cycling, while a fraction with low or no detectable enrichment (f_{C4}~0) would be interpreted as slow cycling or passive, respectively (Poeplau and Don, 2013b).

For the purposes of the comparison, we identified 11 sites, mostly agricultural long-term experiments throughout Europe, with a vegetation change from pure C3 to pure C4 vegetation, and a C3 reference site that had never been cropped with C4 plants. The C4 plants cultivated after the C3-C4 vegetation change were either maize (*Zea mays,* L.) or miscanthus (*Miscanthus* spp.). Sampling depth varied between 0-5 and 0-20 cm. In a

coordinated campaign in spring and summer 2015, a number of soils were sampled with a spade to a depth of approximately 5 cm. Other soils were sampled from archives or separated from regular samplings, which explains the variation in sampling depth. Spatial variability was not important in this study, so only one large composite sample (several kilograms) of each soil was taken during the sampling campaign. All samples were dried at 60°C and sieved to 2 mm.

From among the 11 C3-C4 vegetation change experiments identified, we decided to restrict the study to three experiments in order to keep the total number of samples to be fractionated by each laboratory at a manageable size. To select the three most suitable soils, we determined bulk C and N using an elemental analyzer (LECO TrueMac, St. Joseph, MI, USA) and $\delta^{13}C$ using an isotope ratio mass spectrometer (IRMS) (Delta Plus, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an elemental analyzer (CE Instruments FLASH EA 1122 NA 1500, Wigan, UK). Soil texture was determined with a laser diffraction particle size analyzer (Beckham-Coulter LS 13 320 MW, Brea, CA, USA). Sample preparation consisted in organic matter digestion in hydrogen peroxide followed by chemical dispersion in sodium hexametaphosphate. The selection criteria were: i) a strong C4 signal, i.e., a large difference in $\delta^{13}C$ value (>2‰) between the reference and C4 plots and ii) a range in soil texture across the three selected soils. Furthermore, in order to facilitate isotopic analysis, we excluded any soil that contained carbonates. Finally, two German experiments, Braunschweig (BS) and Rotthalmünster (RT), and one French long-term experiment, Le Closeaux (CL), were selected (Table 1). At BS, a long-term miscanthus experiment was established in 1993 on a sandy Arenosol (according to FAO classification), previously used as grassland. The C3 reference sample was taken from a directly adjacent permanent grassland soil. At RT, two treatments in a long-term experiment on a silt loam (Haplic Luvisol) were sampled: i) continuous wheat and ii) continuous maize. Maize cultivation started in 1979. The long-term experiment at CL is located on a loamy soil (Utric Cambisol) in the park of Versailles castle in Paris. Soils under continuous maize since

1993 and under continuous winter wheat were sampled. However, we discovered that the C3 reference soil at CL was contaminated with C4-derived C. This contamination must have occurred recently, since those fractions considered fast cycling and young were most contaminated. Furthermore, contamination was not observed in earlier studies at the site (Derrien et al., 2006; Fernández-Ugalde et al., 2016). As a consequence, we were not able to apply the concept described earlier using the C3 reference soil at CL. Instead, we used the averaged $\delta^{13}C$ values obtained for the same fraction in each of the two other soils. Those values were highly correlated between the two soils, with R^2 of 0.71 and root mean square error (RMSE) of 0.82. Slightly higher enrichment of ^{13}C in the RT soils was observed (Fig. S1). However, since the regression line and the 1:1 line were parallel with no siginificant difference between slopes, there was a similar shift for all fractions. We thus concluded that the average of BS and RT would be a good approximation of the uncontaminated C3 reference soil at CL in this study where we used the differences between fractionation methods. However, this increased the uncertainty of the two-pool mixing model for CL and the results obtained should be interpreted with caution. Moreover, it should be noted that f_{C4} (Eq. 1) can only be used under steady-state conditions to directly calculate decomposition rates and turnover rates, i.e., when carbon inputs and SOC stocks do not change after vegetation change (Derrien and Amelung, 2011). This was the case for RT and CL (Table 1), while SOC stock at BS increased under miscanthus vegetation. Comparison of turnover rates between the sites was thus hampered by the fact that the BS soil under miscanthus most likely received higher carbon inputs than the C3 reference soil. Consequently, f_{C4} could not be taken as an absolute measure of turnover rates in the BS soil. However, the comparison of f_{C4} values across fractions and fractionation methods was not influenced by these differences between sites.

Participating laboratories were asked to perform a fractionation method established in their laboratory on triplicate samples of each soil (n=18). The bulk samples of all soils were divided using a rotating sample divider

(PT100, Retsch, Haan, Germany) and requested amounts were sent to the participants.

2.2 *Fractionation methods*

There was no systematic a priori method selection but all currently used fractionation methods were welcome to become part of the fractionation trial. However obvious redundancies were avoided. The ensemble of participating methods might thus well reflect the currently prevailing view on SOC stabilization (Lützow et al., 2006). A total of 19 laboratories using 20 methods participated in the experiment. Physical fractionation methods (n=13) were most common (Table 2).

The physical fractionation methods used in the different laboratories can be divided into: i) aggregate size fractionation, whereby undispersed soils (<2 mm in this case) are wet-sieved, ii) particle size fractionation, whereby soils are dispersed before wet-sieving, and iii) density fractionation, which separates a 'light fraction' that consist mostly of 'free', particulate organic matter from more 'heavy' fractions that mostly contain OM that is bound to soil minerals.whereby free organic particles are separated from occluded organic particles and SOC attached/adsorbed to minerals in dispersed and/or undispersed soil samples.

Dispersion, i.e., disruption of aggregates to varying degrees, was achieved by different techniques, including ultrasonication, shaking with glass beads, or addition of hexametaphosphate (HMP), in the different laboratories. Several combinations of these approaches exist, with a combination of particle size and density fractionation being the most common among participants in this study (Table 2). Most often, a sodium polytungstate (SPT, $Na_6H_2W_{12}O_{40}$) solution with a density of >1.6 g cm^{-3} is used as heavy liquid for separation. Water (density 1 g cm^{-3}) wa used for density fractionation in one fractionation method.

Chemical fractionation methods can be divided into extraction, hydrolysis, and oxidation. Extraction or hydrolysis, as a means to isolate organic matter with different chemical composition and/or functionality, is

performed with water, hydrofluoric acid (HA), tetrasodium pyrophosphate ($Na_4P_2O_7$) or sulfuric acid (H_2SO_4). Sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) are used for oxidation, which is performed to mimic enzymatic breakdown of SOC.

Although all of the methods listed in Table 2 may have been developed for different purposes and to answer different research questions, the operationally defined thresholds are often comparable across methods. For example for density fractionation, the most often chosen density cut-off is between 1.6 and 1.85 g cm^{-3} (Griepentrog and Schmidt, 2013). Furthermore, the size classes of aggregates and particles often resemble boundaries between texture classes in the respective classification systems. For example, a size cut-off of 50-63 µm lies near the boundary between fine silt and coarse silt or silt and sand and a cut-off of around 200-250 µm lies at the boundary between silt and sand or fine sand and coarse sand. The exact boundaries often depend on the prevailing soil classification in the country of method origin. Methods Par+Den1, Par+Den4 and Com2 have not been published, but exact protocols of all methods can be found in the supplementary material of this publication and the web-guidelines for fractionation (www.somfractionation.org). Methods Par1, Par2 and Che2 are reduced in complexity compared with the original publications. For method Par1, the two size fractions are usually further separated using NMR spectroscopy (Baldock et al., 2013). In the present study, we were thus only able to analyze two fractions. Method Par2 resembles only the physical fractionation part of the original procedure (Lopez-Sangil and Rovira, 2013). In the original procedure for method Che2 (Rovira et al., 2012), several fractions are isolated by means of acid hydrolysis. Since we were not able to measure these freeze-dried extracts in the IRMS due to the high salt and very low C content, we determined the f_{C4} of a total extractable fraction arithmetically, by subtracting C4-carbon and total C in the residual fraction from C4-carbon and total C in the bulk soil. These methods might therefore have performed better if isotopic data on the total spectrum of intended fractions had been available.

2.3 Criteria to assess the performance of fractionation methods

Range and distribution of turnover rates

As a measure of the ability of a method to isolate fractions with distinct turnover rates, we considered the differentiation in f_{C4} values across fractions as the most important indicator. This differentiation was expressed with the simplest indicator, which was the range of f_{C4}values:

$$Range_{f_{C4}} = f_{C4_{max}} - f_{C4_{min}},$$

(Eq. 2)

where $f_{C4_{max}}$ is the maximum f_{C4} value of a fractionation method and $f_{C4_{min}}$ is the corresponding minimum f_{C4} value. Thereby, $Range_{f_{C4}}$was calculated for each replicate of a given fractionation method and averaged. Figure 2 presents a conceptual example of three methods with varying $Range_{f_{C4}}$. Besides a generally large differentiation in f_{C4}, it is also desirable to achieve a uniform spread among the varying f_{C4} values of fractions, in order to avoid fractions that are similar or equal in turnover rates. As an example, schemes 2 and 3 in Figure 2 show an equal range in turnover times, while scheme 3 has two fractions with similar f_{C4} values. To calculate a simple penalty term for *redundancy* in turnover rates, we first ordered the fractions of each method by descending f_{C4} and calculated the distances between two consecutive fractions. We then calculated the coefficient of variation of all distances within one method (CV_{dist}) as follows:

$$CV_{dist} = \frac{SD_{dist}}{Mean_{dist}} \times 100$$

(Eq. 3)

$$SD_{dist} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}\left(\left(f_{C4_{i+1}} - f_{C4_i}\right) - \left(\frac{\left(f_{C4_{i+1}} - f_{C4_i}\right) + \left(f_{C4_{i+2}} - f_{C4_{i+1}}\right) + \ldots + \left(f_{C4_n} - f_{C4_{n-1}}\right)}{n-1}\right)\right)^2}$$ (Eq. 4)

where SD_{dist} is the standard deviation of all distances and n is the number of fractions.

Similar distances between two consecutive fractions, i.e., a constant spread throughout the range of yielded f_{C4} values, would thus result in a low CV_{dist} (scheme 2 in Figure 2). A relatively high CV_{dist} is in turn an indicator of redundant fractions in a particular fractionation method and might help to identify methods that could potentially be simplified. It should be noted, however, that this measure applies exclusively to the parameter turnover rate, as highlighted in this study. There may be fractions that have a similar turnover rate but differ greatly in other functions, thereby justifying their separation.

Recovery and reproducibility

Recovery and reproducibility of total C are very important aspects to consider when assessing the performance of a fractionation method. Average C recovery for each method and soil was calculated by adding up the measured C masses in each fraction and dividing the sum by the C mass measured in the bulk soil. The CV was used to quantify the variability in total C mass ($CV_{carbon\ mass}$) and in the $\delta^{13}C$ of the three laboratory replicates of each fraction ($CV_{\delta 13C}$) as a measure of reproducibility. A minimum average CV of all fractions of the respective method was considered favorable.

Distribution imbalance

A large variation in f_{C4} could also occur with an unequal distribution of C in different fractions. Especially in agricultural soils, different sizes of fractions with distinct ecological properties are somewhat inevitable and this is not a problem per se. However, in certain circumstances it might become important, for instance if fraction size limits the possibility for further analyses of the fraction. In addition, when the majority of C is stored in a fraction that has a medium turnover rate, while fractions representing active and passive SOC pools are very small, f_{C4} of the bulk SOC might be equally informative, e.g., for initializing a model. Hence, we

calculated the variability in fraction sizes based on the relative proportions of total SOC in each fraction. The indicator was expressed as standard deviation ($SD_{fraction\ size}$), which was to be minimized. Here the CV could not be used, because the mean value of the relative fraction size is determined by the number of fractions isolated.

Workload

Although workload is not a methodological criterion as such, it can be important to consider when selecting a method. Therefore, all participating laboratories were asked to state the average net time needed to fractionate one sample. The range of reported values was 0.5 hour to 27 hours. In view of the fact that the absolute value of this parameter is influenced by numerous factors and is difficult to estimate, we decided not to present these absolute values directly, but used them in the evaluation scheme as described below.

Evaluation scheme

For decision support and robust and comprehensive method comparison, we developed an evaluation scheme. Two different steps were involved: First, we rated each method for each criterion (differentiation in turnover rate, redundancy, distribution imbalance, reproducibility, recovery, and workload) using the three groups ‘very good’, ‘good’, and ‘fair’. Thresholds for separating the methods into the three groups were defined for each indicator individually, and are given at the bottom of each column in the evaluation matrix. This was done to avoid predefining a fixed number of methods in each group. We then derived an overall rating for each method by linear transformation of the six continuous variables to a number between 1 and 100, where 1 was the ‘worst’ and 100 the ‘best’ result. We then asked all participants, before they had seen the results, how they would weight each criterion to calculate an overall performance index. Based on the average values of this survey, we calculated the overall performance index (OPI) as:

$$OPI = 0.32 \times Differentiation \in turnover\ rate + 0.07 \times Redundancy + 0.2 \times Recovery + 0.2 \times Rep$$

For the OPI, we used the upper and lower quartile to allocate methods to each group. We used a correlation matrix to evaluate the relationships between these performance indicators and the number of fractions. This was done to elucidate potential intercorrelations between indicators and the effect of number of fractions on the performance of a method.

2.4 Statistical analysis

We used linear mixed effect models to test the average difference in $Range_{f_{C4}}$ between the method types (aggregate size classes, particle size classes, density classes, aggregates+density, particles+density, chemical and combined) across all soils for significance at $p<0.05$. Method nested in soil were used as random effects. Further linear mixed effect models were used to evaluate the potential influence of dispersion method on the quality criteria recovery and reproducibility. Thereby, treatment (C3/C4) nested in soil were used as random effects. This was done using the package nlme (Pinheiro et al., 2009) in the environment of the statistical software R (R Development Core Team, 2010). Normal distribution of the residues was visually assessed and confirmed using Q-Q-plots. Slopes of the linear regression and the 1:1 line in Figure S1 were compared using ANCOVA in R.

2 Results

3. 1 Distribution of carbon in different fractions

The average recovery of C mass after fractionation was 95%. Recovered SOC was distributed in the isolated fractions as shown in Figure 2. The aggregate size fractionation methods (Agg1, Agg2, Agg+Den1, Com3) had the most balanced distribution of SOC across fractions, while fractionation by particle size, density, or chemical treatment resulted in less balanced SOC distributions. Particles <53 µm, i.e., the silt and clay fraction, stored the majority of SOC (up to 85%) when soils were dispersed and separated by particle size (e.g., methods Par1, Par2, Com4). Likewise, heavy fractions with density >1.65 g cm^{-3} contained the majority of SOC (e.g.,

Den1, Den2, Den3, Par+Den1, Par+Den2). In the CL soil, up to 95% of the total recovered SOC was stored in this heavy fraction (Den1). Oxidation with H_2O_2 or NaOCl left only a small resistant fraction (Che1, Com2, Com3, Com4), while the residual fraction after water, K_2SO_4, or $Na_4P_2O_7$ extraction was much larger than the extracted fractions as such (Che2, Com5). Water and K_2SO_4-extractable SOC only accounted for about 1% of total SOC (Com1, Com5). Despite the differences in soil texture, these patterns were observed in all three soils.

3.2 Differentiation in proportion of C4-derived carbon

The enrichment of C4-derived C after 22-36 years in the isolated fractions of the three soils ranged from 0-76% and was distributed as shown in Figure S2. In the sandy BS soil a skewed distribution was detected, with very few fractions containing less than 30% C4-carbon. For RT and CL the distribution was approximately normal, indicating more continuous enrichment of C4 in the isolated fractions of these two soils.

On average, we found a significant effect of method type on variation in the proportion of C4-derived C ($Range_{f_{C4}}$) (Fig. 3). The combined methods yielded the highest variation in the proportion of C4-derived C. Particle-size class fractionation with density separation yielded significantly higher $Range_{f_{C4}}$ values than the other method types. The lowest average $Range_{f_{C4}}$ was found with the methods isolating different aggregate size classes. In all three soils, isolating particle size classes and density classes tended to yield higher $Range_{f_{C4}}$ values than aggregate fractionation (Fig. 4A, 4C, 4E).

Figures 4B, 4D and 4F depict $Range_{f_{C4}}$ for all methods individually. For BS and RT, method Com5 yielded the highest $Range_{f_{C4}}$. The density fractionation method with the highest $Range_{f_{C4}}$ was method Den2, which isolated five different fractions of varying density. Among the particles+density fractionations, method Par+Den5, in which density fractionation is performed after size separation in both the fine and coarse fractions, yielded the highest $Range_{f_{C4}}$ of all soils.

3.3 C4-derived carbon accumulation in individual fractions

Figure 5 shows the average f_{C4} values in each fraction for all 20 methods and for all three soils. The highest accumulation of C4-derived C, up to 76%, was observed in light particulate organic matter fractions that were extracted using density fractionation (all 'Den' methods) or electrostatic attraction (Com5). In the following, those fractions are referred to as the light fraction (LF), while the non-floating counterpart is referred to as the heavy fraction (HF). Size fractionation of LF always resulted in a strong spread in f_{C4} values, underlining the importance of particle size for the turnover of LF (Agg+Den1, Par+Den2, Par+Den4, Par+Den5, Com5). In contrast, the separation into free and occluded LF without size separation did not yield as pronounced differences (Den3, Par+Den1, Com4). Moreover, in method Par+Den4, the size fractionation of occluded LF (oLF) yielded two fractions that differed strongly in f_{C4}, where oLF >20 µm tended to be relatively more enriched in C4 than free LF, whereas the f_{C4} of oLF <20 µm was similar to that of the mineral soil <20 µm. As shown in Figure 8, LF separated well from HF in terms of f_{C4} only in coarse fractions (50-2000 and 250-2000 µm), while in finer fractions no clear differences between LF and HF were apparent. Comparison of methods Par1 and Den3 provides a further example that size is more important to differentiate turnover rate than occlusion: Par1 only separated two size fractions without any density fractionation, whereas Den3 performed density fractionation after stepwise dispersion, isolating a free LF, an occluded LF, and a HF. In all three soils studied, the variability of f_{C4} was higher for Par1 than for Den3 (Figs. 2, 5). Even method Den1, which isolated only LF and HF, omitting oLF, resulted in a higher variation in f_{C4} than Den3 in two out of the three soils (Fig. 4).

Across all soils, the lowest f_{C4} was found in NaOCl-resistant SOC fractions (Com3, Com4) and fractions with density >2.6 g cm^{-3} (Den2). Isolation of fractions with f_{C4} values <0.2 was only possible by means of extraction, oxidation, or use of a very dense solution.

Extraction with water (Com4, Com5) or K_2SO_4 (Com1) did not yield fractions with comparatively large accumulation of C4-derived C as LF. In those methods that also isolated HF and LF, the water- or K_2SO_4-extractable C was closer to HF than to LF in terms of accumulation of C4-derived C.

Regarding size fractionation, the separation of particles or aggregates at 50/53 µm (the border between silt and sand; methods Agg1, Agg2, Par1, Agg+Den1, Par+Den1, Par+Den2, Par+Den3, Par+Den5, Com4, Com5) or 20 µm (Par+Den4, Com2) always yielded strong contrasts in f_{C4}, while separation into coarse sand (>250 µm) and fine sand (~53-250 µm) or similar boundaries (Agg1, Agg2, Agg+Den1, Par+Den4, Par+Den5) tended to be less effective. In general, f_{C4} increased with particle size and the difference in f_{C4} between coarse and fine particles tended to be higher in particle size fractionation methods than in aggregate size fractionation methods (Figures 3, 4, 6), due to the fact that macroaggregates also contain microaggregates and fine particles.

3.3 Further quality criteria and their interactions

The total C recovery of all methods was high, with an average of 95% (Table 3). The minimum recovery rate for a fractionation method was 88%, while the maximum was 101%. The average variability in C mass ($CV_{carbon\ mass}$) observed across the three replicates in each fraction was 16% and this indicator for reproducibility was found to increase with number of fractions (Fig. 7). Method Com2 had the highest $CV_{carbon\ mass}$ (35%, Table 3), which can be explained by the very high variability in the liquid fraction. Variability in $\delta^{13}C$ was generally low, with an average $CV_{\delta 13C}$ of 1.5%. Recovery was found to negatively correlate with $CV_{\delta 13C}$ as the second reproducibility indicator, which highlights the importance of high recovery. Losing SOC may lead to shifts in the isotopic signature of a fraction and of the bulk soil. The dispersion method significantly affected recovery and reproducibility (Fig. 8). Despite a slightly higher average number of fractions per method, dispersion with HMP had the highest recovery and the lowest variability in carbon mass of each fraction across the three

replicates (Fig 8B, 8C). Only the methods without dispersion had a higher reproducibility, while this comparison is clearly biased by the much lower average number of fractions of the undispersed methods (Fig 8A). Glass bead dispersion methods had a significantly higher recovery than ultrasonic dispersion methods, while no clear difference in reproducibility was detected between those two.

Distribution imbalance, i.e., unequal distribution of SOC in different fractions, was negatively correlated with number of fractions, indicating that the risk of isolating fractions that differ in size is higher when fewer fractions are isolated. It is therefore undesirable to isolate fractions that are of very different sizes, since this leaves the majority of SOC in one single fraction.

The most complex methods with many fractions (Agg+Den1, Com3, and Com5) have partly isolated functionally redundant fractions, as revealed by CV_{dist} (Fig. 5, Table 3). Redundancy in f_{C4} was highest in Com5 (Table 3) and was positively correlated with number of fractions (Fig. 7). In methods Agg+Den1 and Com3, soil is wet-sieved to different aggregate size classes, which are then dispersed and further fractionated. In method Agg+Den1, the HF <53 µm isolated from aggregates >250 µm did not differ from the corresponding fraction isolated from aggregates <250 µm with regard to C4-derived C accumulation. Similarly, NaOCl treatment of fractions <53 µm (Com3) yielded similarly high f_{C4} values when these particles were extracted from aggregates or isolated outside aggregates (Fig. 5). Finally, workload was also found to strongly correlate with number of fractions (Fig. 7).

4 Discussion

In this study, the aim was to identify the method, or type of method, that is most effective in isolating fractions that differ strongly in terms of SOC turnover in agricultural soils. The general performance pattern of the 20 methods investigated did not differ greatly between the three soils studied. As indicated in the material and methods section, the higher C4-

derived C accumulation in BS soil compared with RT and CL soils is related to the fact that the BS soil accumulated SOC after a vegetation change, and should thus not be interpreted as faster turnover of SOC.

The 20 different fractionation methods compared covered a wide spectrum of principles and complexity, and were designed to elucidate soil organic matter characteristics and dynamics that might be linked to turnover or not. As expected, the performance of these methods in isolating fractions with varying turnover also differed greatly, which enabled re-evaluation of current views on the importance of different SOC stabilization mechanisms and facilitated selection of suitable fractionation methods, at least for temperate agricultural soils. Due to the diversity of methods and the inevitably unbalanced design of the study, we were not able to derive statistical evidence for all arguments presented. However, the results of the study allow the following interpretations:

i) No isolated SOC fraction matches the most active pools in turnover models

The detected range of f_{C4} (0-0.76) in the isolated fractions based on a C3-C4 vegetation change confirms the validity of using C4 accumulation as an indicator of the turnover of fractions, an approach used in numerous previous studies (Lefroy et al., 1993; Balesdent et al., 1998; Del Galdo et al., 2003; Poeplau and Don, 2013b). However, similarly to findings in other studies of about the same duration (Gregorich et al., 1997), no fraction entirely consisting of C4-derived C was isolated, even 22 and 36 years after the shift from C3 to C4 vegetation. Thus, not even the most labile fractions isolated by means of this ensemble of 20 different fractionation methods could be directly linked to the fresh plant litter C pools in biogeochemical models, which usually have a turnover of one to several years only (Coleman and Jenkinson, 1996; Andrén and Kätterer, 1997). Light-fraction SOC is commonly assumed to be the fraction that turns over most rapidly (von Lützow et al., 2007). While this general trend was confirmed in the present study in relative terms, our results challenge the assumption that this light-fraction SOC is equivalent to the fresh plant

litter C pools in turnover models (Zimmermann et al., 2007b). This is surprising, since litter bag studies usually find very rapid decomposition of root or shoot litter (0.5-5 years), depending on litter quality and environmental conditions (Andren and Paustian, 1987; Silver and Miya, 2001) until an asymptotic value of typically <10% of the initial mass is reached (Adair et al., 2008). In soils (outside litter bags), this relatively more recalcitrant part of litter is likely to be incorporated into other fractions, besides remaining as free or occluded LF (Cotrufo et al., 2015). The fact that a considerable proportion of C3-derived C was detected in the LF of all three soils analyzed in the present study might indicate that density fractionation is not a suitable means to isolate fractions that consist only of the most recently added plant material and microbial necromass. Thus, short-term plant interactions and microbial turnover processes cannot be captured by means of density fractionation. Flotation of mineral particles or aggregates with associated SOC that cycles much more slowly cannot be avoided (Cerli et al., 2012), which might be one important factor explaining the mixed signal in the LF. This is confirmed by the low average C content (29%) in all coarse fLF. The expected C content would have been 45-50% for fresh plant litter. Cerli et al. (2012) found considerable amounts of mineral particles even in density fractions <1.4 g cm^{-3} of forest soils, which indicates that this problem is not specific to agricultural soils. However, even the isolated coarse LF that contained >40% C did not consist entirely of new, C4-derived carbon. Another factor that might explain the persistence of C3-derived carbon in LF would be the presence of pyrogenic carbon (Soong and Cotrufo, 2015). However, it was not analyzed in this study and would require future investigation.

In most fractionation methods, water-extractable C is viewed conceptually as a labile SOC fraction, due to its high bioavailability and the perception that primarily young and uncomplexed organic matter can be dissolved (Zimmermann et al., 2007b; Zhao et al., 2008). At the same time, it is well known that dissolved organic C (DOC) is generally variable in terms of biological availability. In agricultural soils, it has been found that only 14-25% of the collected DOC is labile (Kalbitz et al., 2003). Poeplau et al.

(2017) followed the distribution of SOC in different fractions along a gradient of extreme natural soil warming and found that in a nearly unvegetated +40°C warming treatment, SOC declined by up to 80% over six years, with the proportion of DOC to total SOC being highest in these highly warmed plots. This indicates that DOC is not primarily litter-derived, but is in equilibrium with the mineral-associated fraction through sorption and desorption. Moreover, Poeplau and Don (2013a) found the DOC fraction to be slightly less sensitive to land use change than the bulk SOC. This agrees well with the results of the present study, since in all methods which extracted SOC either with water (Com4, Com5) or a K_2SO_4 solution (Com1), the extracted fraction displayed similar C4-derived C enrichment to the mineral-associated SOC. These findings suggest that the concept of extracting labile SOC by water does not hold, at least for the arable soils studied here. This confirms the findings of a literature survey by von Lützow et al. (2007), who concluded that it is difficult to use DOC as a SOC fraction with a distinct turnover rate. Nonetheless, due to its high mobility, DOC is a key functional pool which is useful for understanding and modeling the vertical distribution of SOC within the soil profile (Neff and Asner, 2001).

ii) SOC resistant to oxidizing reagents has the slowest turnover

The largest variability in SOC turnover rates was found for fractionation methods applying chemicals to extract or oxidize C in order to isolate a resistant fraction (Figs. 3, 4, 6). This supports findings in other studies (Marzaioli et al., 2010). Oxidizing reagents are applied to mimic strong, enzymatic attack. Research has been conducted to evaluate the efficiency of different chemical treatments to isolate stable SOC (Helfrich et al., 2007), and ^{14}C age has been found to strongly increase in residual fractions, by up to 2000 years compared with the bulk soil (Kleber et al., 2005; Helfrich et al., 2007). Interaction with poorly crystalline minerals and polymeric metal species has been identified as the most important mechanism for oxidation resistance of SOC (Kleber et al., 2005; Mikutta et al., 2005; Mikutta and Kaiser, 2011). The abundance of oxalate-soluble iron (Fe) and aluminum (Al) explains most of the variability in stable SOC

observed in several soils (Kleber et al., 2005). Accordingly, Percival et al. (2000) highlighted Al and Fe content as more important for SOC stabilization than clay content. Thus, despite existing criticism of equating chemical stability with the biological stability of SOC (Leifeld and von Lützow, 2014), the results of this study confirmed that SOC resistant to chemical oxidation had the slowest turnover. Moreover, fractionation methods applying only chemical oxidation or hydrolysis techniques resulted in good separation of fractions with different turnover (Fig. 3). In line with other studies (Poeplau and Don, 2013b, a), the results also showed that those fractions cannot directly be linked to an 'inert' pool as used in many C turnover models (Zimmermann et al., 2007b), because turnover of SOC within two or three decades was detectable.

iii) SOC attached to particles with density >2.8 g cm^{-3} cycles as slowly as oxidation-resistant SOC

The fractionation method described by Sollins et al. (2009) (Den2) is based on sequential density fractionation using salt solutions of four different densities, and it performed well in isolating SOC fractions of different turnover rates. The difference in C4-derived C accumulation between particles with density 2.4-2.8 g cm^{-3} and particles with density >2.8 g cm^{-3} regarding C4-derived C accumulation was particularly high in all three soils (Fig. 5). Particles with density >2.8 g cm^{-3} displayed f_{C4} values of 0.09 (BS), 0.13 (RT), and 0 (CL), which was within the range of values observed for NaOCl-resistant SOC. In the original paper (Sollins et al., 2009), mineralogical analyses showed that this fraction mainly consists of pedogenic oxides and ferromagnesian primary minerals. This underlines the importance of mineralogy, and especially Fe-oxides, for SOC stabilization. However, this fraction was also very small, comprising on average only ~2.5% of total SOC. It is likely that oxidation-resistant SOC yielded a similar fraction to this high density separation, which is also confirmed by similar C contents in these fractions, ranging from 0.2-0.5% in the high density fractions and from 0.2-1.1% in oxidation resistant fractions. As further evidence of this, both fractions had very low f_{C4}, yet

also relatively negative ^{13}C values, in the C3 reference soils (data not shown). This is well in line with observations by Zimmermann et al. (2007a), who did not find strong enrichment in ^{13}C after NaOCl oxidation in C3 soils, and by Sollins et al. (2009), who found no further ^{13}C enrichment at densities >2.4 g cm^{-3}. The latter hypothesized that the C in this fraction was relatively young at the time of attachment to the surfaces, but resisted further microbial breakdown afterwards.

iv) Particle size separates the light fraction of different turnover rates better than incorporation into aggregates

The youngest and fastest cycling SOC is associated with particulate organic matter or LF, with density <1.65 g cm^{-3} or less (Fig. 4). This material is not attached to minerals and consists to varying extents of relatively fresh plant or animal residues. In any concept of SOC formation, whether it is humification, selective preservation, progressive decomposition, or the soil continuum model (Lehmann and Kleber, 2015), this C fraction is at the very beginning of SOC formation. The C4-derived C accumulation of up to 76% within two decades, as well as $\delta^{13}C$ values <-28‰ in the C3 reference soils, which resembles the common $\delta^{13}C$ value of C_3 plants, confirmed the status of LF as young and fast cycling. To isolate LF in which the C is somewhat older than in coarse fLF, two different approaches are applied: Size fractionation of the LF or isolation of occluded LF SOC. The results of this study suggest that separation into particle size classes is more successful than separation into free and occluded LF (Fig. 7). This is in line with findings by Balesdent (1996) showing that coarse (200-2000 µm) LF material comprises 15% slow-cycling SOC, while fine LF material (50-200 µm) comprises about 35% slow-cycling SOC.

As an example, the Par+Den4 method separated occluded LF into >20 µm and <20 µm. The >20 µm occluded LF had a higher f_{C4} value than the free LF in all three soils, while the <20 µm occluded LF showed almost identical f_{C4} values with <20 µm HF. Thus, occluded LF is apparently extremely inhomogeneous and LF particle size seems to be an important predictor

for turnover rate. This challenges the concept behind classical fractionation methods isolating free and occluded LF (Golchin et al., 1994b), although it has been concluded that the isolated occluded organic matter is in various stages of decomposition (Golchin et al., 1994a). Method Par1 (Sanderman et al., 2014), representing a very simple particle size fractionation that separates particles >50 µm from particles <50 µm, gave higher variability in f_{C4} than Den3, a classical method isolating fLF, oLF, and mineral-associated SOC (Golchin et al., 1994b). Therefore, particles >50 µm had similarly high f_{C4} values to the fLF fractions isolated by other methods, indicating that a majority of the carbon stored in sand fractions is fLF.

The relatively low f_{C4} in fine LF is most likely caused by admixture with mineral-associated SOC. In all three methods which applied size fractionation to LF, the fine LF had a C content ranging from 10-20%, while the coarse LF had an average C content of 29%. This clearly indicates the presence of minerals in fine LF. Conducting density fractionation after size fractionation, as done in methods Par+Den5, Com4, and Com5, might solve this problem, at least for coarse LF (>250 µm), since sand grains are less likely to float or stick to plant tissues than silt and clay particles.

v) Separating silt and clay from sand-sized fractions yields strong contrasts in turnover rate

Aggregate size class and particle size class fractionation methods were most effective in achieving high contrast in C4-derived C enrichment when separating silt plus clay from sand sized particles (cutoff at 50, 53, or 63 µm). Further separation of the sand fraction (at 250 µm) was less efficient. This is in line with findings in other studies (Eusterhues et al., 2003; Flessa et al., 2008) and highlights the importance of fine microaggregates and organo-mineral interactions for SOC stabilization, from which the concept of C saturation evolved (Hassink, 1997). In this challenged concept, the amount of SOC that can be stored in a soil is limited by the amount of silt and clay minerals. In agricultural soils, this fraction can store up to 90% of the total SOC (Flessa et al., 2008; Ghafoor et al., 2017).

vi) Fractionation of aggregate size classes is less effective than fractionation of particle size classes

The hierarchical organization of soil aggregates is postulated to play a crucial role for SOC stabilization (Elliot, 1986) (Jastrow, 1996). In this hierarchy, fine microaggregates (<53 µm) are proposed to act as a physical barrier between decomposer and substrate, and thus protect SOC from rapid mineralization (Six et al., 2002b). Given this hierarchical organization of aggregates, any macroaggregate contains subunits of microaggregates and, consequently, a certain proportion of stabilized C, which is determined by factors such as land use and soil mineral characteristics. This is reflected in the high degree of redundancy in fractions in the methods Agg+Den1, Com3 and Com5, as well as the low range of f_{C4} in the methods Agg1 and Agg2. At the same time, aggregates can have a turnover on a timescale of months (Monnier, 1965; Virto et al., 2010). If material is found to be occluded within an aggregate at the time of fractionation, it is not possible to determine how long ago that occlusion occurred. Moreover, plant roots (primarily root hairs) can penetrate aggregates (Rasse et al., 2005), or play an important role in aggregate formation via mucilage, root exudates, and fungal hyphae (Traore et al., 2000; Rillig, 2004). The fact that aggregates and especially microaggregates are beneficial for SOC stabilization might thus not exclude the possibility of aggregate re-formation and fresh SOC input into aggregates, resulting in a constant mix of young and older SOC. Also in other soils, aggregates have been shown to undergo rapid turnover (Puget et al., 2000; Virto et al., 2010). Consequently, the separation into different aggregate size classes was less effective in separating fractions with a distinct turnover rate than fractionation into dispersed particle size classes (Figs. 3, 4, 5). In the task of deriving SOC fractions with homogeneous turnover rates, aggregate size class fractionation will by definition fail (Christensen, 2001). However, due to the important role of aggregates for SOC sequestration and other soil functions, aggregate fractionation methods are useful to investigate the effects of disturbances such as land

use change, tillage, or warming on SOC dynamics and soil structure (Six et al., 1999; Poeplau et al., 2017).

Lessons learnt for the future development or choice of fractionation methods

The ability to isolate SOC fractions that differ in turnover rate tended to increase with complexity of the method. The fractionation method Com5, published by Kaiser et al. (2016), which isolates 10 fractions with a combination of density, aggregate size and extraction by water and $Na_4P_2O_7$, had the highest average $Range_{fC4}$. However, at the same time, the method showed the highest redundancy across fractions and among the lowest recovery and reproducibility (Table 3). Thus, in addition to the high workload, the most complex methods have serious drawbacks. Based on the evaluation table (Table 4) and the overall performance index, methods Par1, Par+Den5, Che2, Com3, and Com4 performed best and had considerably fewer fractions (two to six). Among these, Com3, which isolated six different fractions, had the highest redundancy. It can be concluded that three to five fractions might be sufficient to isolate SOC pools with a wide range of turnover rates. This resembles the number of pools in the most frequently used turnover models, such as RothC and Century (Parton et al., 1988; Coleman and Jenkinson, 1996). In the particular case of Com3, the two NaOCl-resistant fractions could be pooled.

Mineralogy was found to be very important for SOC stabilization, as inferred from: i) the observed difference in turnover rates for the clay+silt versus sand fractions and ii) low f_{C4} in oxidation-resistant SOC and density fractions >2.8 g cm^{-3}. Water extraction does not yield a fraction that differs much from the bulk soil with respect to turnover rate. In contrast, size fractionation is useful for separating SOC with different turnover rates. Separation into particles coarser or finer than ~50 µm is particularly effective in this regard. This also holds true for LF, for which the finest fractions showed similar turnover to HF. Thus, based on the results of this comprehensive comparison, a combination of size fractionation after

preceding dispersion of aggregates, separation of coarse LF by density fractionation, and a further density or chemical fractionation step to isolate a highly refractory SOC fraction can be recommended, based on the results of this comprehensive comparison. When only two fractions with varying turnover rates shall be isolated, e.g. due to time shortage, size separation into sand and silt+clay-sized particles might be most effective (method Par1).

It has been highlighted before, that sample dispersion is a critical step in the majority of SOC fractionation procedures (Schmidt et al., 1999; Kaiser and Asefaw Berhe, 2014; Poeplau and Don, 2014). The investigated methods in the present study used three different dispersion measures: chemical dispersion with HMP as well as physical dispersion with ultrasonic and glass beads. Although a direct comparison of these measures was not possible, due to the variability of methods used, we found that HMP dispersion performed slightly better than the physical measures regarding recovery and reproducibility. This might be an indication, that chemical dispersion is more exact and less prone to errors such as loss of material during change of vessels or incomplete dispersion due to e.g. variation in energy output during sonication. However, a more systematic approach is necessary to confirm these results.

The increase in f_{C4} over time is not a linear process and does not follow the same dynamic in each fraction (Balesdent, 1996). Thus the observed differences in f_{C4} values between fractions are specific to the moment of sampling and cannot be extrapolated to longer (or shorter) periods of time. Additionally, we only investigated temperate agricultural soils. The performance of the assessed methods might be different in soils under other land use types or climate conditions, or derived from different parent material. The performance might further vary between laboratories. Keeping these limitations in mind, the evaluation in Table 4 should be interpreted with care. However, we did not detect fundamental differences between the three soils studied regarding the performance of the methods. This led us to make similar recommendations for each of the

soils investigated, which may be useful for future fractionation studies. Finally, the average values for each quality criterion are given in Table 3, so the overall performance index (*OPI*) could be recalculated with adjusted weightings whenever needed. Further information on the original purpose of different fractionation methods is given in the internet-based guidelines (www.somfractionation.org).

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List of tables

Table 1. Details of the three experimental sites: Mean annual temperature (MAT, °C), mean annual precipitation (MAP, mm), sampling depth (Depth, cm), soil organic carbon content of the C3 reference soil (SOC_{C3}, %) and the C4 soil (SOC_{C4}, %), proportions of sand, silt and clay [%] and soil pH(H_2O), years under C4 vegetation [yrs], C4 plant species, and the proportion of C4-derived carbon (f_{C4}) in the bulk soil.

Soil	ID	Depth	MAT	MAP	SOC_{C3}	SOC_{C4}	sand/silt/clay$_{C3}$	sand/silt/clay$_{C4}$	pH_{C3}	pH_{C4}	Years under C4	C4 Plant	$\delta^{13}C_{C3}$	$\delta^{13}C_{C4}$	f_{C4}
Braunschweig	BS	0-5	8.8	620	1.55	1.83	67/29/4	64/31/5	6.25	6.31	22	Miscanthus	-28.8	-19.6	0.54
Rotthalmünster	RT	0-5	8.7	886	1.4	1.31	17/75/8	14/78/8	6.23	6.27	36	Maize	-27.3	-20.34	0.39
Les Closeaux	CL	0-20	10.6	651	1.28	1.3	34/59/7	35/58/7	6.02	6.10	22	Maize	-25.4	-23.29	0.3

Table 2. List of 20 fractionation methods applied at participating laboratories, with class and type of method, original reference, citation index of the source (in Web of Science), number of fractions isolated, sample mass [g], dispersion method, applied density [g cm^{-3}], chemicals used for oxidation/extraction/hydrolysis (Ox/Ex/Hyd), and size ranges of the isolated particles or aggregates [μm].

ID	Class	Type	Reference	Cited	n	Sample mass	Dispersion	Density	Ox/Ex/Hyd	Sizes
Agg1	Physical	Aggregates	Elliot et al., 1986*	903	3	5				0>53>250>2000
Agg2		Aggregates	Six et al., 2000*	843	3	50				0>53>250>2000
Par1		Particles	Sanderman et al., 2014	7	2	10	HMP			0>50>2000
Par2		Particles	Lopez-Sangil and Rovira, 2013*	13	4	15	Ultrasonic			0>20>50>200>2000
Den1		Density	Sollins et al., 1984*	188	2	10		1.6		
Den2		Density	Sollins et al., 2009*	97	5	16	Ultrasonic	1.6,2,2.4,2.8		
Den3		Density	Golchin et al., 1994	336	3	20	Ultrasonic	1.6		
Agg+Den1		Aggregates+Density	Six et al., 1998	622	10	30	HMP	1.85		0>53>250>2000
Par+Den1		Particles+Density	Six et al., 1998*	622	4	20	HMP, glass beads	1.85		0>53>2000
Par+Den2		Particles+Density	Shaymukhametov et al., 1984	13	5	10	Glass beads	2		0>20>1000
Par+Den3		Particles+Density	Diochon et al., 2016	2	5	30	Glass beads	1.7		0>5>53>250>2000
Par+Den4		Particles+Density	Steffens et al., 2009*	42	5	30	Ultrasonic	1.8		0>20>2000
Par+Den5		Particles+Density	Balesdent et al., 1987	422	5	30	Glass beads	1		0>50>200>2000
Che1	Chemical	Oxidation	Mikutta et al., 2006	253	2	3			NaOCl, HA	
Che2		Hydrolysis	Rovira et al., 2012*	14	4	0.5			H2SO4	
Com1	Combined	Particles+Extraction	Unpublished	0	5	6	Glass beads		K2SO4	0>63>2000
Com2		Particles+Oxidation	Leifeld and Kögel-Knabner, 2001	54	3	30	Ultrasonic		H2O2	0>20
Com3		Aggregates+Oxidation	Six et al., 2000	843	7	20-33	HMP		NaOCl	0>53>250>2000
Com4		Particles+Density+Oxidation	Zimmermann et al., 2007*	135	5	30	Ultrasonic	1.8	NaOCl	0>0.45>53>2000

Com5	Aggregates+Particles+ Density+ Extraction	Kaiser et al., 2016*	3	10	5	Ultrasonic	1	Na4P2O7	0>0.45>53>250

*=Method is slightly modified from the original.

HMP = hexametaphosphate, HA = hydrofluoric acid

Table 3. Different quality criteria describing the performance of each fractionation method: Average differentiation in turnover rates expressed with $Range_{fC4}$ (the range of the average f_{C4} values of all fractions); average redundancy expressed with CV_{dist} (calculated as described in Eq. 3); average recovery; average reproducibility expressed with the two indicators coefficient of variation of the carbon mass (CV_{Cmass}) and coefficient of variation of the $\delta^{13}C$ values across the three laboratory replicates of each fraction; average distribution imbalance expressed with the standard deviation of the average fraction sizes (carbon mass), and distribution imbalance of carbon across fractions for the 20 methods. For explanation of method ID codes, see Table 2.

ID	Differentiation in turnover rates	Redundancy	Recovery	Reproducibility		Distribution Imbalance
	$Range_{fC4}$	CV_{dist}	% of bulk SOC	CV_{Cmass}	$CV_{\delta 13C}$	$SD_{fraction\ size}$
Agg1	0.08	50	97	8	1.4	16
Agg2	0.22	71	99	10	1	16
Par1	0.27		101	4	0	40
Par2	0.22	63	96	13	1.7	34
Den1	0.17	76	95	8	2.2	55
Den2	0.41	81	89	29	2	22
Den3	0.15	72	91	15	1.9	36
Agg+Den1	0.3	84	101	25	0	10
Par+Den1	0.25	98	89	8	2.8	28
Par+Den2	0.28	73	88	17	1.8	16
Par+Den3	0.31	53	96	15	1.5	20
Par+Den4	0.33	85	92	27	1.8	19
Par+Den5	0.45	86	101	15	1.4	28
Che1	0.25	97		11	1.3	38
Che2	0.3			16	0.6	14
Com1	0.29	13	101	35	1.9	35
Com2	0.36	54		6	0.8	28
Com3	0.33	85	98	16	1.2	12
Com4	0.49	67	97	19	1.2	25
Com5	0.56	153	89	25	3.2	20
Average	**0.301**	**76**	**95**	**16**	**1.5**	**26**

Table 4. Evaluation of the 20 different fractionation methods using different criteria and an overall performance indicator (OPI). Methods in bold gave the best overall rating.

ID	Differentiation	Redundancy	Recovery	Reproducibility	Distribution Imbalance	Workload	OPI
Agg1	Fair	Very good	Very good	Very good	Very good	Intermediate	Good
Agg2	Fair	Good	Very good	Very good	Very good	Low	Good
Par1	**Good**	**Very good**	**Very good**	**Very good**	**Fair**	**Low**	**Very good**
Par2	Fair	Good	Very good	Good	Good	Intermediate	Good
Den1	Fair	Good	Very good	Good	Fair	Low	Fair
Den2	Very good	Good	Good	Fair	Good	High	Good
Den3	Fair	Good	Very good	Good	Fair	Low	Fair
Agg+Den1	Good	Good	Good	Good	Very good	Intermediate	Good
Par+Den1	Good	Fair	Good	Good	Good	Low	Fair
Par+Den2	Good	Good	Good	Good	Very good	High	Fair
Par+Den3	Good	Very good	Good	Good	Good	High	Good
Par+Den4	Good	Good	Good	Fair	Good	High	Fair
Par+Den5	**Very good**	**Good**	**Very good**	**Good**	**Good**	**Low**	**Very good**
Che1	Good	Fair	Very good	Very good	Fair	Low	Good
Che2	**Good**	**Very good**	**Very good**	**Very good**	**Very good**	**Low**	**Very good**
Com1	Good	Very good	Very good	Fair	Fair	Low	Good
Com2	Good	Very good	Very good	Very good	Good	High	Good
Com3	**Good**	**Good**	**Very good**	**Good**	**Very good**	**Low**	**Very good**
Com4	**Very good**	**Good**	**Very good**	**Good**	**Good**	**High**	**Very good**
Com5	Very good	Fair	Good	Fair	Good	High	Good
	Fair <0.25	Fair >86	Good <95%	*	Fair >34	Low <4h/sample	
	Very good	Very good	Very good >94%		Very good	High >7h/sample	

>0.41	<63	<16

*Reproducibility was calculated as a mean of $CV_{\delta 13C}$ and $CV_{carbon\ mass}$, both distributed between 1 and 100.

Figure captions

Figure 1: Example of three soil fractionation methods (methods 1-3) with varying ranges of f_{C4} between soil fractions (indicator for differentiation of turnover rates) and varying CV_{dist} (indicator of redundancy in turnover rates). Each bar represents one fraction.

Figure 2: Average proportion of total carbon (C) in each fraction for the Braunschweig (BS), Rotthalmünster (RT), and Le Closeaux (CL) soils, depending on the method used (for explanation of ID codes, see Table 2) with standard error (bars). LF = light fraction; HF = heavy fraction; agg = aggregates; rem. = remaining; ext = extractable. Units in the fraction descriptions are µm, or g cm^{-3} in the case of density fractions.

Figure 3: Average range in proportion of C4-derived carbon ($Range_{fC4}$) for all soils and method types, with standard error (bars). Different letters indicate significant differences at $p<0.05$ and numbers at the bottom of each bar indicate the number of fractionation methods in each type. The number of observations used in the statistical model is thus nine fold the given number (three soils, three replicates).

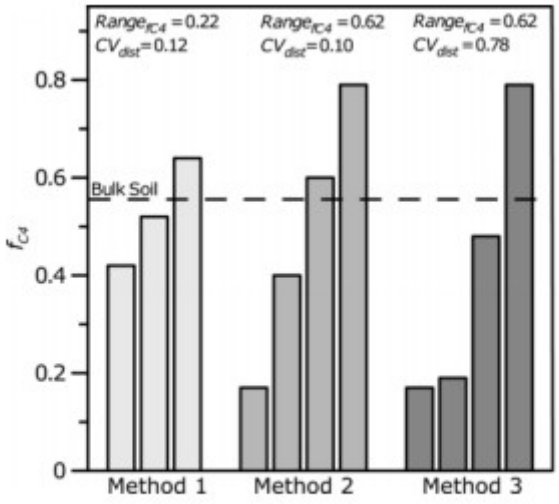
Figure 4: Range of values obtained for the proportion of C4-derived carbon across SOC fractions ($Range_{fC4}$) for: (A, C, E) the Braunschweig (BS), Rotthalmünster (RT), and Le Closeaux (CL) soils, separately averaged for all method types; and (B, D, F) the 20 different individual methods, reported by their ID code (for explanation see Table 2). Error bars indicate standard errors.

Figure 5: Average f_{C4} values for all fractions and soils investigated with standard error (bars). For explanation of method ID codes, see Table 2. LF = light fraction; HF = heavy fraction; agg = aggregates; rem. = remaining; ext = extractable. Units in the fraction descriptions are µm, or g cm^{-3} in the case of density fractions.

Figure 6: Average proportion of C4-derived C (f_{C4}) values for different size classes (µm, x-axis) and for differently isolated fractions. LF = light fraction; HF = heavy fraction.

Figure 7: Correlation matrix for the different quality criteria and number of fractions. The colors of the circles relate to the strength and direction of the correlation shown on the bar next to the y-axis. Size of the circles also relates to the strength of the correlation.

Figure 8: A) Average number of fractions per method, B) average recovery [%], C) average coefficient of variation of carbon mass across replicates, D) average coefficient of variation of $\delta^{13}C$ values across replicates for different soil dispersion methods. Error bars indicate standard errors and different letters indicate significant differences.



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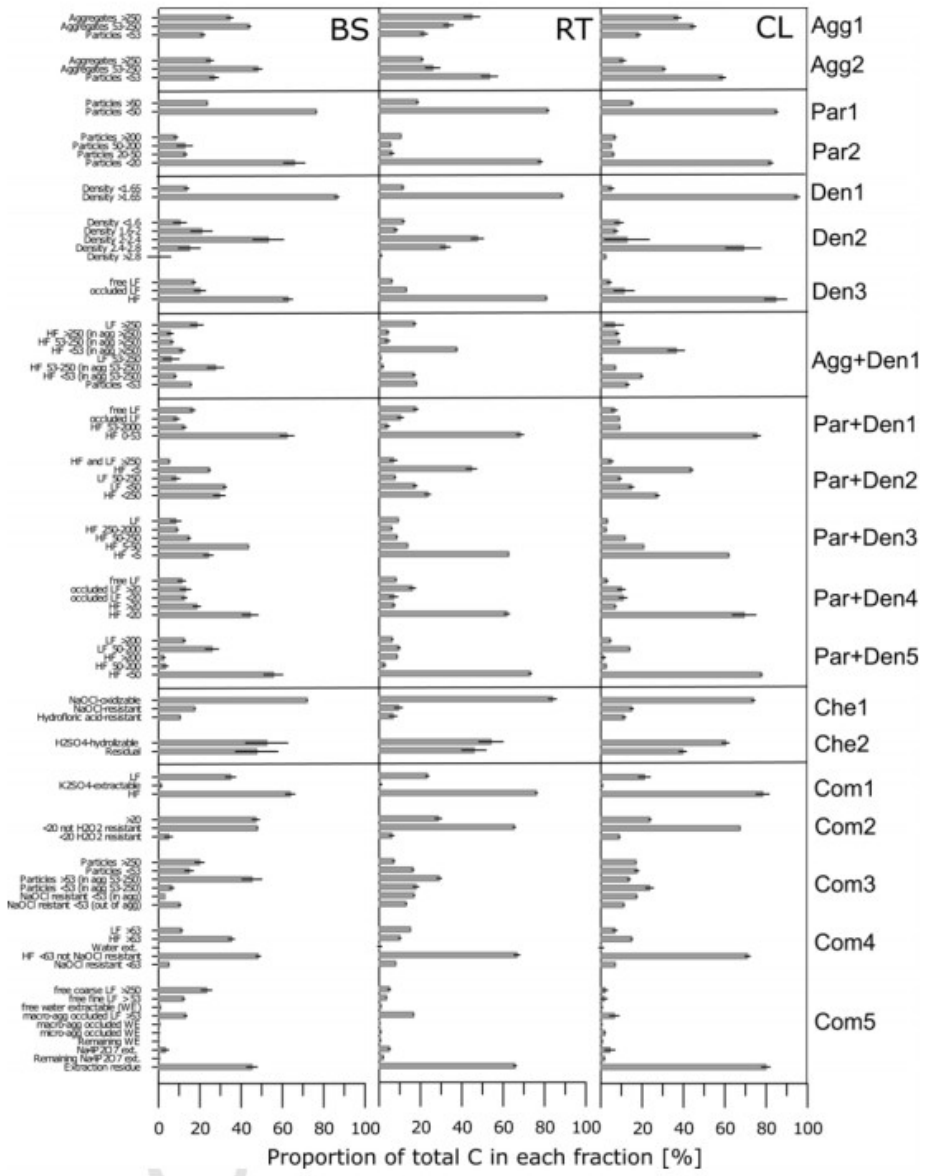

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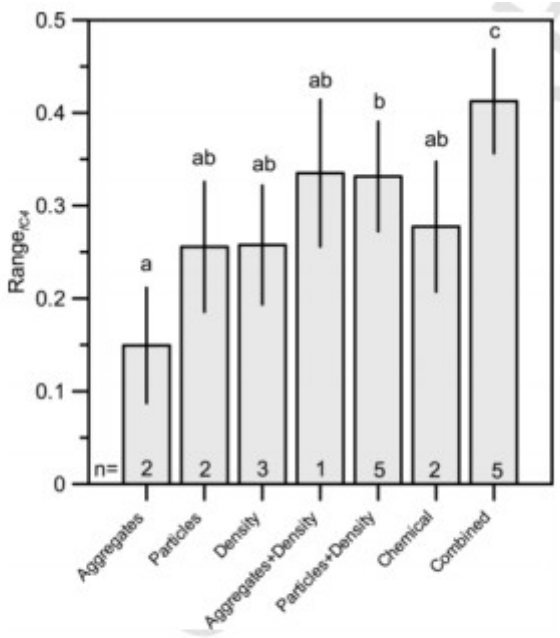

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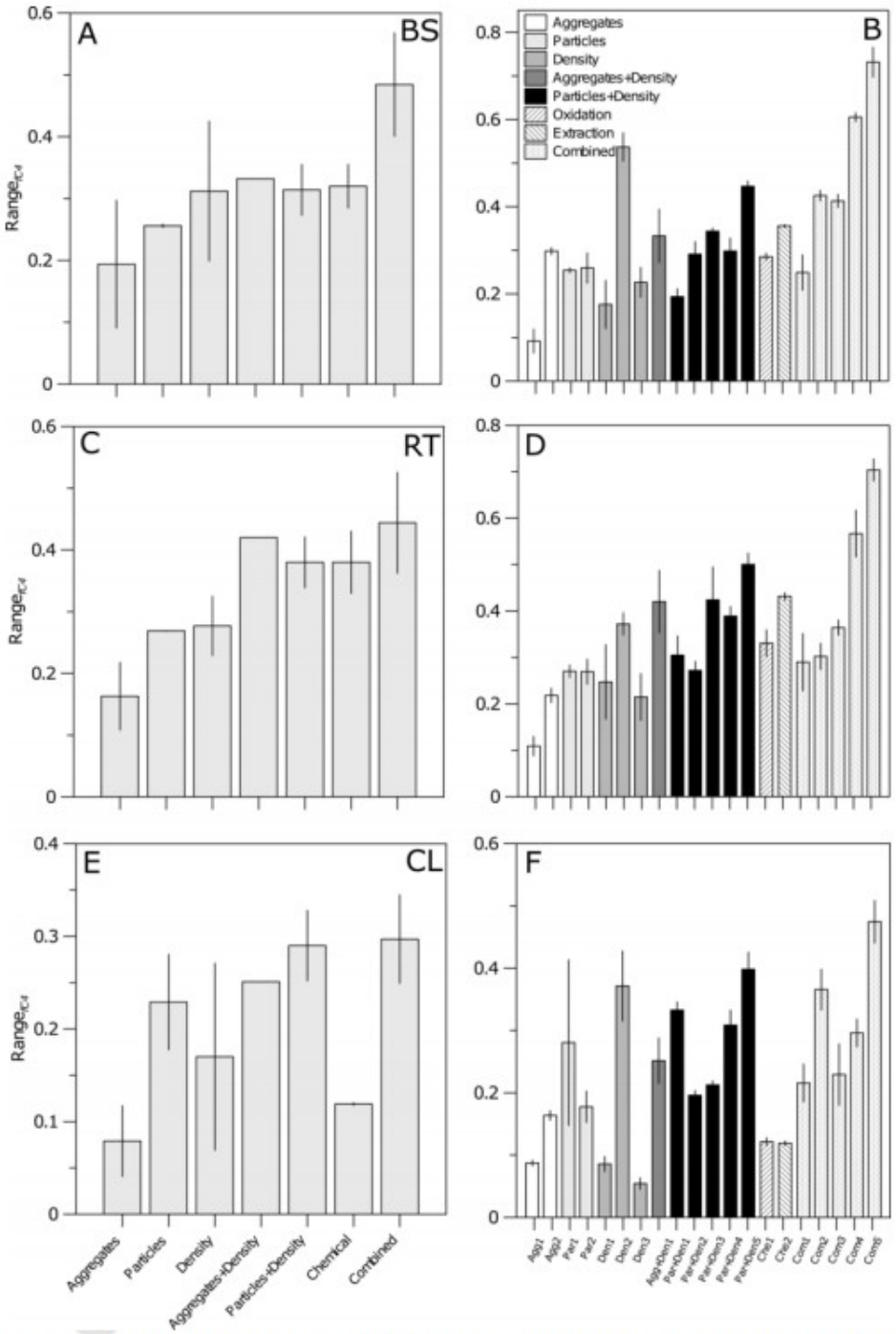

Fig. 4. Range of values obtained for the proportion of C4-derived carbon across SOC fractions ($Range_{fC4}$) for: (A, C, E) the Braunschweig (BS), Rotthalmünster (RT), and Le Closeaux (CL) soils, separately averaged for all method types; and (B, D, F) the 20 different individual methods, reported by their ID code (for explanation see Table 2). Error bars indicate standard errors.

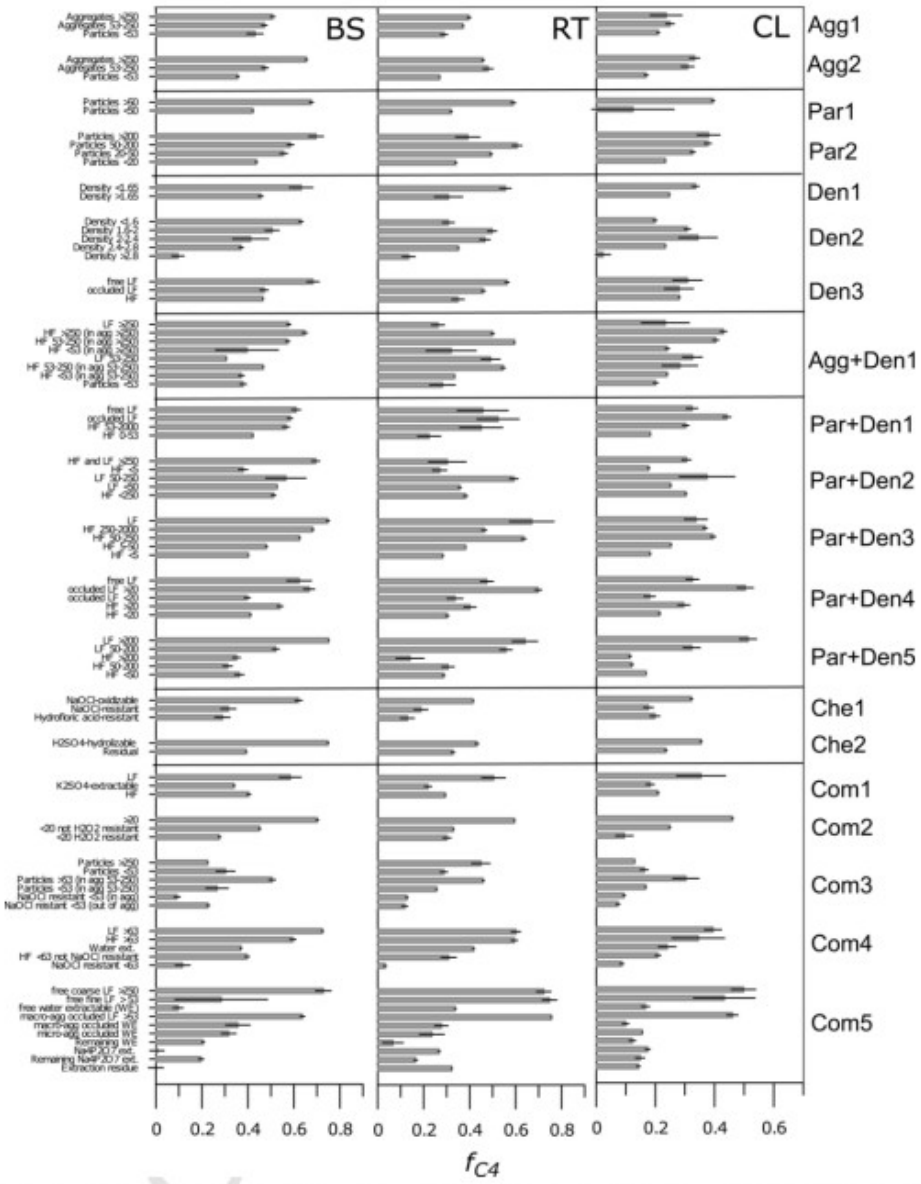

Fig. 5. Average f_{C4} values for all fractions and soils investigated with standard error (bars). For explanation of method ID codes, see Table 2. LF = light fraction; HF = heavy fraction; agg = aggregates; rem. = remaining; ext = extractable. Units in the fraction descriptions are μm, or g cm^{-3} in the case of density fractions.

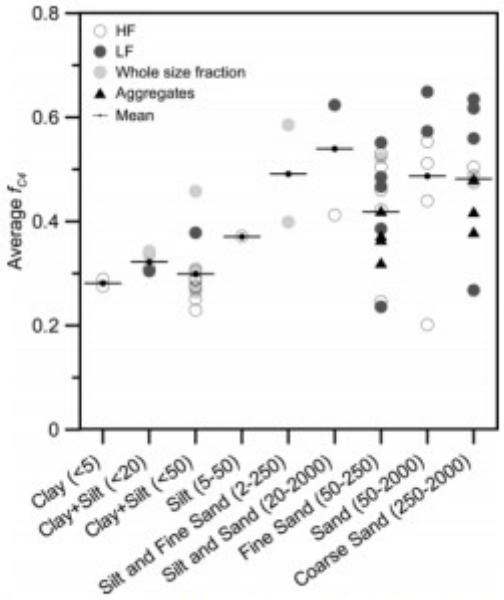

Fig. 6. Average proportion of C4-derived C (f_{C4}) values for different size classes (µm, x-axis) and for differently isolated fractions. LF = light fraction; HF = heavy fraction.

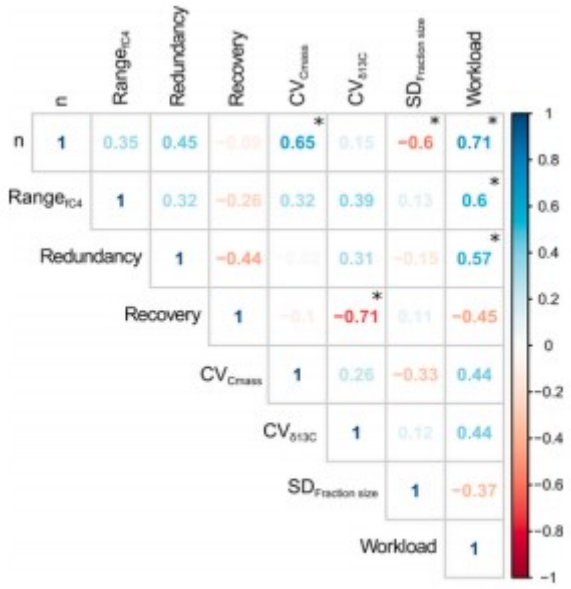

Fig. 7. Correlation matrix for the different quality criteria and number of fractions. The colors of the numbers relate to the strength and direction of the correlation and the asterisks indicate statistical significance ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

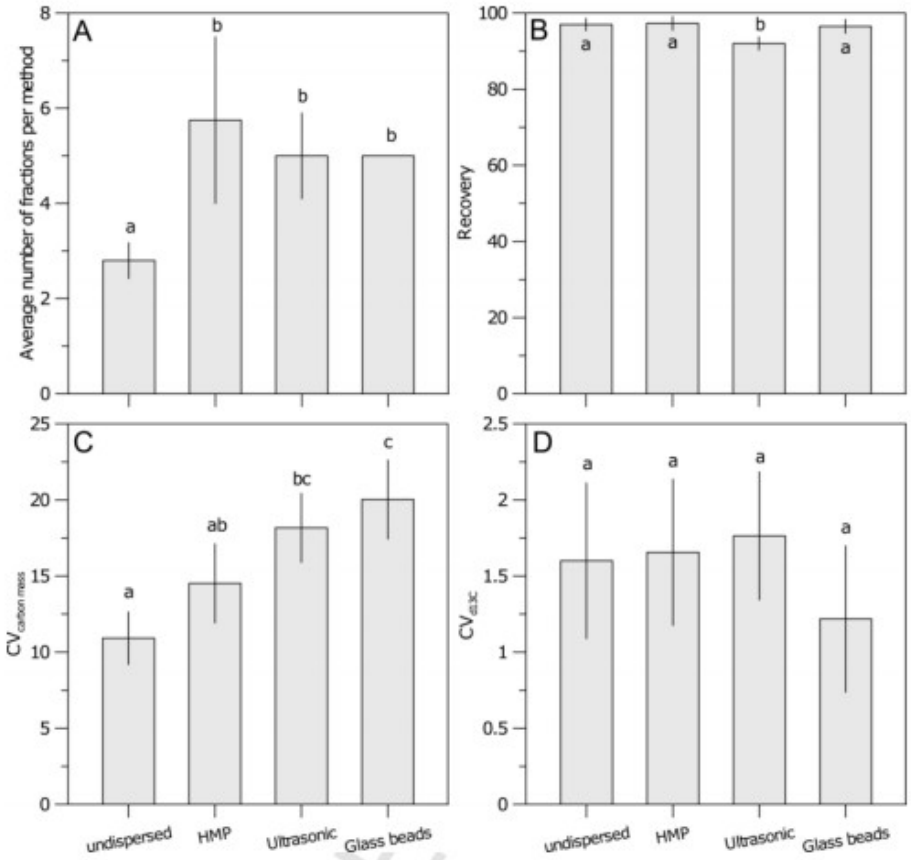

Fig. 8. A) Average number of fractions per method, B) average recovery [%], C) average coefficient of variation of carbon mass across replicates, D) average coefficient of variation of $\delta^{13}C$ values across replicates for different soil dispersion methods. Error bars indicate standard errors and different letters indicate significant differences.