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Reproducibility of a soil organic carbon fractionation method to derive RothC carbon pools

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Summary

Fractionation of soil is undertaken to isolate organic carbon with distinct functional properties, such as stability and turnover times. Soil organic carbon (SOC) fractionation helps us to understand better the response of SOC to changes in land use, management or climate. However, fractionation procedures are often poorly defined and there is little information available on their reproducibility in different laboratories. In a ring trial, we assessed the reproducibility of a SOC fractionation method introduced by Zimmermann *et al.* (2007). The isolated fractions were linked to the model pool sizes of the Rothamsted carbon model (RothC). We found significant differences between six laboratories for all five defined fractions in three different soils with coefficients of variation ranging from 14 to 138%. During ultrasonic dispersion, the output power (energy per unit time) was identified as an important factor controlling the distribution of SOC within these five fractions, while commonly only the output energy is standardized. The amount of water used to wet-sieve dispersed soil slurry significantly influenced the amount of extracted dissolved organic carbon (DOC). We therefore suggest using a fixed amount of power for ultrasonic dispersion (20 W) and a minimum amount of water for wet sieving (2000 ml). RothC pool sizes were predicted from the measured fractions and compared with RothC equilibrium pool size distributions. This model initialization using measured SOC fractions, however, led to an over-estimation of stable RothC SOC pools when compared with pool size distributions derived from RothC equilibrium runs under a bare fallow soil model simulation. To improve the isolation of particulate organic matter from stable mineral-bound organic matter, we suggest that the density should be increased from 1.8 to 2.0 g cm^{-3} in the density fractionation step. We formulated a modified fractionation procedure, which aims specifically to enhance reproducibility across laboratories and to improve the match of the isolated SOC fractions with RothC's SOC pools.

Introduction

Soil organic carbon (SOC) consists of several components, which differ in their physico-chemical properties and hence their degree of stabilization and turnover time (Bol *et al.*, 2009). Active SOC fractions are considered to respond relatively quickly to changes in land use, climate or management, while other fractions are hypothesized to be more stable or even inert (Krull & Skjemstad, 2003). The distinct turnover times range from less than one year to thousands of years. The composition of SOC with its individual fraction size distribution thus determines its sensitivity towards any kind of disturbance (Conant *et al.*, 2004).

Modelling of SOC dynamics plays a key role in large-scale estimates and predictions of SOC stock changes by accounting for the variability of soils, climate and land use (Paustian, 2001). Starting in the late 1970s, several multi-compartment models have been developed (Jenkinson & Rayner, 1977). The majority of these models are based on the partition of SOC into different functional pools with distinct first-order kinetics and climatic and edaphic

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rate modifiers (Paustian, 2001). For almost two decades many attempts have been made to develop SOC fractionation methods to correlate laboratory-derived carbon fractions with functional model pools in order to initialize the models or to validate the outputs (Motavalli *et al.*, 1994). Elliott *et al.* (1996) stated that only models that can be validated by measurements are reliable enough to predict SOC changes. However, it is challenging to achieve a good match of operationally defined SOC fractions with the conceptional model pools, because the turnover of a specific fraction depends on its chemical and physical properties. Smith *et al.* (2002) stated that a measured fraction is only equivalent to a model pool if it is unique and does not contain 'sub-fractions' with contrasting properties.

Existing fractionation methods can be divided into two major groups: physical and chemical fractionation, which are based on different scopes regarding the mechanisms of SOC stabilization. Physical fractionation is based on the assumption that the association with soil particles and aggregates plays the key role in SOC stabilization and thus different SOC fractions are isolated by different degrees of disaggregation, dispersion, density fractionation and particle size separation (Amelung & Zech, 1999; Christensen, 2001; Six *et al.*, 2001). In contrast, chemical fractionation methods are based on the assumption that the chemical composition of each fraction determines its stability and thus its turnover time (Blair *et al.*, 1995; Helfrich *et al.*, 2007). Chemical fractions are obtained by extraction of SOC in different solutions, the hydrolysable nature of SOC with water or acid and the resistance to oxidation. In several studies a combination of physical and chemical fractionation has been used (Trumbore *et al.*, 1989; Leifeld & Kogel-Knabner, 2001). The idea is to separate younger SOC from mineral-bound SOC by density or particle size fractionation and these physical fractions are chemically treated to separate further fractions of different stabilization degrees and turnover times. However, the use of loose terms, such as 'recalcitrance' or the vague definitions for fractions such as particulate organic matter (POM) or mineral-associated organic matter (MOM) has confused the discussion and hampered direct comparisons of obtained results (Schmidt *et al.*, 2011). Additionally, fractionation procedures are often poorly defined and there is little information available on their reproducibility in different laboratories. Therefore, there is a need for more standardized methods. A new attempt to combine physical and chemical fractionation has been made by Zimmermann *et al.* (2007). These authors isolated fractions that were related to the modelled pools of the Rothamsted carbon model (RothC) (Jenkinson & Rayner, 1977): these were two active, two slowly cycling and one passive fraction. Meanwhile the method has been used in different studies and has the potential to become a new widely used fractionation method because of its simplicity and good match with the RothC pools when applied to low altitude arable and grassland soils (Dondini *et al.*, 2009; Leifeld *et al.*, 2009a; Xu *et al.*, 2011). However, not all steps of the fractionation procedure are described in detail (Zimmermann *et al.*, 2007). It is therefore likely that each laboratory has developed its own system and optimization, which might in turn lead to significantly different results. Therefore, we set up a ring trial to compare the measured SOC fractions from six different laboratories and relate these to the RothC modelled carbon pools. Primarily, the aim of this work is to identify the most bias-sensitive steps. As a second step we aim to suggest a more standardized fractionation protocol for this method.

Materials and methods

Soils, sample preparation and experimental setting

Three different cropland soils (0–20 cm) with contrasting properties were selected for a ring trial. Soil 'A' is a loamy clay from the experimental farm Kungsängen (University of Uppsala, Sweden), soil 'B' a loamy sand from the agricultural research station Reckenholz (ART, Switzerland) and soil 'C' is a coarse sand from the experimental farm Jyndevad (University of Aarhus, Denmark) (Table 1). A further description of the sites and their soils can be found in Poeplau & Don (2013). The soils have been under permanent crop cultivation for more than 100 years (Soils A and C) and 60 years (site B). The main crops at Kungsängen (site A) were spring barley, spring wheat, winter wheat, ley and oats. At Reckenholz (site B) the crops were winter wheat, maize, potatoes, spring barley and ley and at the Jyndevad (site C) they were spring barley, winter barley, winter rye and oats. At Kungsängen the field received farmyard manure with an average carbon input of 0.4 Mg C ha^{-1} $year^{-1}$. All three soils were free of carbonate. The soils were dried at 40°C, sieved to 2 mm and cleared of visually detectable fine roots. Soil sampling (to a depth of 80 cm), sample preparation and SOC stock calculation are described in detail in Poeplau & Don (2013). To obtain homogenized and reproducible subsamples of 30 g, which is the required amount for the fractionation, we used a sample splitter (RT, Retsch, Haan, Germany).

Table 1 Characteristics of the three soils and site characteristics at the sampling sites, C_{org} concentration, SOC stock (0–20 cm depth), pH (KCl), and clay, silt and sand contents

Soil	Site	Country	C_{org} / %	Bulk density / g cm^{-3}	SOC stock / Mg ha^{-1}	pH(KCl)	Clay %	Silt %	Sand %
A	Kungsängen	Sweden	2.90	1.01	58.3	4.4	38.1	54.4	7.6
B	Reckenholz	Germany	0.95	1.32	25	4.6	14.9	35.7	49.5
C	Jyndevad	Denmark	1.19	1.27	30.3	5.6	4.7	5.7	89.6

The reproducibility was tested on five randomly selected subsamples for each soil. For the soils of sites A and B the coefficient of variation (CV) of the carbon content was 2 and 3% among the subsamples. For site C, which is characterized by a large content of particulate organic matter (POM) but a relatively small content of total SOC (1.2%), there was a CV of 5%, which was, however, considered to be acceptable. Three randomly selected subsamples of each soil (total $n = 9$) were sent to each of the participants in the ring trial. There were six laboratories in the trial at the Thuenen Institute of Climate Smart Agriculture (Germany), Agroscope Reckenholz (Switzerland), INRA, Clermont-Ferrand (France) in cooperation with University of St Andrews (United Kingdom), the Technical University Munich (Germany), the University of New England (Australia) and the University of Aberdeen in cooperation with the Centre for Ecology and Hydrology (CEH) (United Kingdom). The participants were asked to conduct the fractionation as described in Zimmermann *et al.* (2007) and to document every fractionation step in detail. The participating institutes have been made anonymous by randomly numbering (1–6). To avoid an additional instrumental bias, the participants were asked to send the dried fractionated samples back to the Thuenen Institute, where all solid fraction samples were analysed for total carbon and nitrogen by dry combustion in an elemental analyser (LECO TruMac, St. Joseph. Michigan, USA). Only the dissolved organic carbon fraction (DOC) was analysed by each laboratory because of the problems associated with sending frozen soil extracts.

SOC fractionation

The fractionation (Zimmermann *et al.*, 2007) comprised the following steps: (i) ultrasonic dispersion with 22 J ml^{-1} to disperse labile macroaggregates, (ii) wet-sieving over a 63-µm sieve to separate the coarse, the fine and the dissolved fraction, (iii) density fractionation with a sodium polytungstate solution with a density of 1.8 g cm^{-3} to separate a light and a heavy fraction within the coarse fraction; and (iv) a sodium hypochlorite (NaOCl) oxidation with a 1-g subsample of the fine fraction to simulate aggressive decomposition. The method isolates the following five fractions: particulate organic matter (POM), dissolved organic carbon (DOC) (both considered to be active C pools), SOC attached to sand grains and in stable aggregates (S+A), SOC attached to silt and clay particles without being chemically resistant (both considered to be slow cycling) and a chemically resistant fraction (rSOC) (Figure 1).

Modelling the pool sizes with RothC and converting fractions to pools

To estimate the SOC pool size distributions for the three soils with RothC, we used the model's inverse approach, which calculates the plant carbon input to achieve the measured total SOC stock at equilibrium. The required input data were thus reduced to the following: precipitation, temperature, open-pan evaporation and plant cover on a monthly basis, clay content and amount and time of organic manure application. From these data the monthly C input was estimated with RothC and the pool of inert organic matter (IOM) was calculated by the model and using the equation proposed by Falloon *et al.* (1998). Both sets of information are required to initialize an equilibrium run, which we subsequently conducted. The assumption that all three soils were in an approximate equilibrium at the time of sampling could be made because they have been under cultivation without major management changes for 60 (site B) or more than 100 (sites A and C) years.

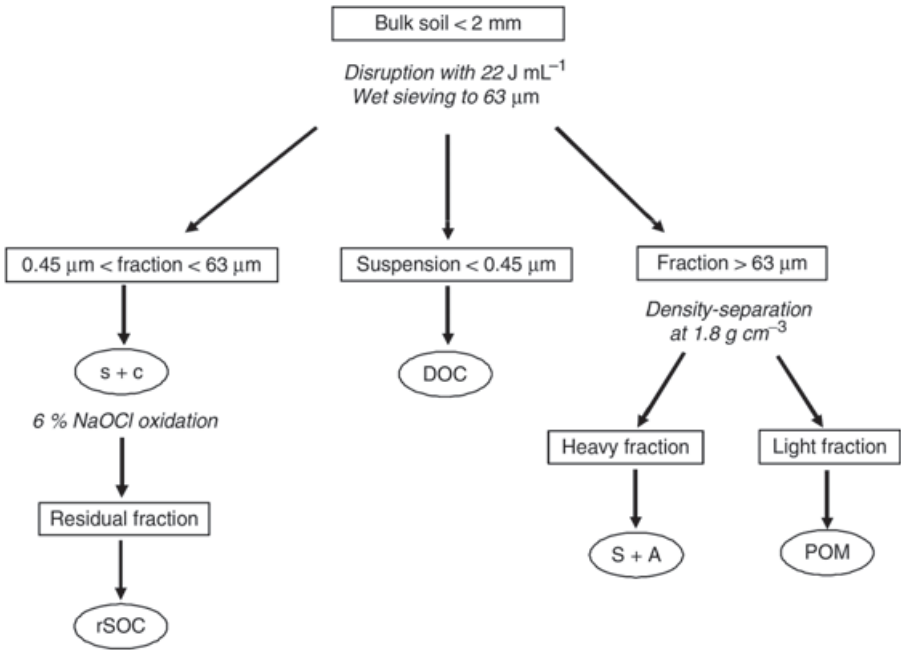

Figure 1 Soil carbon fractionation scheme after Zimmermann *et al.* (2007).

To compare the effect of pool size distribution, either derived from the equilibrium runs or from soil fractionation, on the long-term fate of simulated SOC we converted the measured SOC fractions into pools according to the procedure described by Zimmermann *et al.* (2007) (Figure 2). The splitting ratios for decomposable plant material (DPM) and resistant plant material (RPM) (DPM/RPM) as well as microbial biomass (BIO) and humified organic matter (HUM) (BIO/HUM) were derived from the modelled equilibrium situation at each site, in which the size of each pool was predicted. We also used the measured pools from the six laboratories and the RothC modelled pools to initialize a short model run in RothC to predict the effect of the individual pool size distributions on relative SOC stock depletion. The sensitivity of the total SOC stock, as determined by the different pool size distributions, is thus assessed. To do so, an unrealistic scenario of 40 years bare fallow without any C input was assumed. In the following this modelling exercise will be referred to as the 'bare fallow simulation'.

A posteriori fractionation experiments

We found a large variability in quantified fractions among laboratories, as well as systematic deviations from the RothC modelled pool size distributions, and therefore we conducted several *a posteriori* fractionation experiments in order to improve the protocol and to verify identified sources of laboratory bias.

Wet sieving and density fractionation. As the amount of extracted DOC was correlated with the amount of water used for wet sieving with a saturation occurring at 2000–3000 ml, we *a posteriori* conducted the wet sieving for all three soils with 3000 ml water. Zimmermann *et al.* (2007) isolated only small amounts of DOC and POM, which resulted in a misfit with the RPM+DPM pools. They proposed that the density of sodium polytungstate (SPT) solution used for density fractionation should be increased to 2.0–2.2. We tested the effect of this increase by conducting additional density fractionations with an SPT solution density of 2.0–2.2. To calculate the amount of POM needed to match the modelled RPM and DPM pools, we used the amount of DOC that was *a posteriori* isolated with 3000 ml water and subtracted this amount from the RPM and DPM pools. We also checked whether the amount of isolated DOC could be increased by hot-water extraction of the suspension after ultrasonic dispersion. After a pretest, in which a sample was extracted for 5, 10, 15, 30 and 60 minutes, we decided to extract the samples for 60 minutes in a water bath.

NaOCl oxidation. Chlorine in NaOCl solution is easily lost, so we varied the concentrations of NaOCl to test this as a potential source of bias and used 3, 4, 5, 6 and 7% NaOCl solutions.

Statistical analysis

To assess whether the size of each fraction is significantly influenced by the laboratory that conducted the analysis and to quantify differences regarding the inter-laboratory variability between the three soils, we conducted linear mixed effect model analyses with crossed random effects. There were obvious differences in carbon content between the three soils, which were therefore accounted for by a fixed site effect. The six

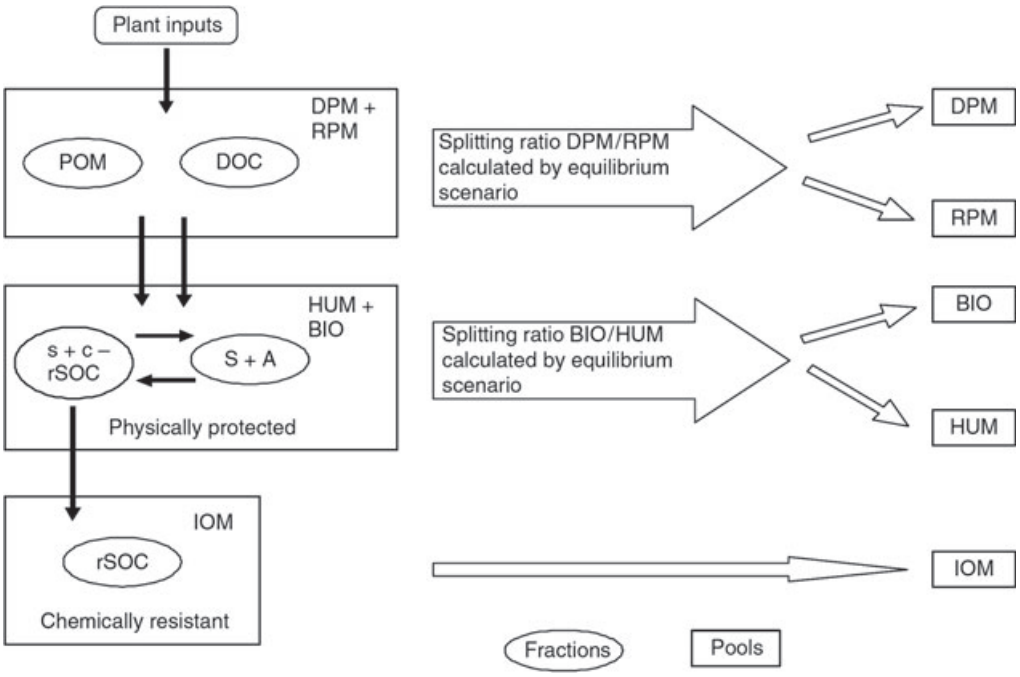

Figure 2 Concept of converting measured carbon fractions to RothC pools after Zimmermann *et al.* (2007).

laboratories were considered as a random selection of a larger number of possible laboratories and therefore treated as random effects. Similarly, the analysed soil samples constituted a random selection of all possible soil samples. As they were split before sending them to the different laboratories, the samples themselves had to be treated as a random effect in the statistical analysis. Two modifications of this basic model were made: first, the differences in carbon content between the different soils were associated with differences in the within-laboratory variability (variance heterogeneity). The residual variance was therefore chosen to be sample-type specific. Second, we had to assume that the differences between laboratories were sample-type specific, demanding an additional random laboratory x sample interaction to be included in the model. Variance components in this model were estimated with the restricted maximum likelihood method as implemented in the nlme-package of the statistical environment R. Model selection was made by using the Akaike Information Criterion (AIC). The level of significance was set to $P = 0.05$. To quantify in which soil and fraction the laboratory effect was most evident, we calculated the coefficient of variation (CV) for each soil and fraction, as well as a ratio of this CV and the residual error (standard deviation) as revealed by the model, which was recalculated into a 'residual CV' (intra-laboratory variability). The larger this ratio, the larger was the difference between laboratories. A ratio <1 thus indicates that the intra-laboratory variability exceeded the inter-laboratory variability. In addition to the five fractions, we conducted this analysis for the ratio of carbon in the fine fraction ($<63\,\mu m$) (s+c, DOC) and carbon in the coarse fraction ($>63\,\mu m$) (POM, S+A).

Results and discussion

Inter-laboratory variability of SOC fractions

We found a significant difference between laboratories for all fractions and the C($<63\,\mu m$):C($>63\,\mu m$) ratio (Figure 3). The POM fraction had the least variability between laboratories and thus the best reproducibility with a CV of 12.9% for site A, 17.6% for site B and 13.1% for site C (Table 2). Density fractionation, which isolates the POM fraction, can thus be classified as the most robust fractionation step regarding the reproducibility between laboratories. The residual CV of the POM fraction was of the same order of magnitude as the CV between laboratories. For site C it was even larger, resulting in a CV:residual CV ratio of 0.7. This was surprising because we expected the POM fraction to be variably distributed in the different soil samples. However, the largest variability between laboratories was observed for the S+A fraction, with a CV of 126.7% for site A, 86% for site B and 58.3% for site C, which is reflected by the large variability of the C($<63\,\mu m$):C($>63\,\mu m$) ratio and indicates that the first fractionation steps (ultrasonic dispersion and wet sieving) were very sensitive to a laboratory bias (Figure 3, Table 2). The largest reproducibility within laboratories was found for the s+c fraction, with a residual CV of 2.5 for site A, 2.1 for site B and 5.7 for site C. This can be explained by the fact that this fraction contains the largest proportion of the total SOC and is thus more robust against losses during fractionation than smaller fractions. Even at site C, which contained only 10.4% clay and silt, $69 \pm 17\%$ of the total SOC was found in the s+c fraction (data not shown). Laboratories 1–5 isolated an s+c fraction of similar size (mg C), whilst laboratory 6 found a significantly smaller s+c fraction for all three soils (Figure 3). This was different for the rSOC fraction, which was obtained by NaOCl oxidation of 1 g of the s+c fraction. The variability between laboratories was much larger for the rSOC than for the s+c fraction, which indicates that the NaOCl oxidation was more sensitive to bias.

Modelled and measured SOC pools

The variability of the SOC pool size distributions among the six laboratories and the deviations from the corresponding modelled SOC pool size distribution were large for all three soils (Tables 3, 4). The soil with the smallest variability in the total determined SOC stock with a coefficient of variation of 2.6% was the fine textured, carbon-rich soil from Kungsängen (A). Three of the six laboratories (2, 4 and 6) measured a similar pool size distribution to that modelled by RothC for site A (Figure 4a, Table 4), which is reflected in the bare fallow simulation (Figure 4b). Site A lost 40.6% SOC in 40 years with the RothC equilibrium pool size distribution and 36.7, 37.6 and 39.4% SOC using the measured SOC pool size distribution from laboratories 2, 4 and 6. In contrast, with the fractionation data from laboratories 1, 3 and 5, SOC depletions of only 33.8, 31.4 and 30.2% were computed.

The greatest variability in the total measured SOC stock among laboratories (21.8%) was observed in the sandy soil at site C (Table 3). The results of the bare fallow simulation ranged from relative SOC losses of 27.5–50.7%. The average deviation from the bare fallow simulation with the RothC equilibrium pools was also greatest for site C, as revealed by the residual sum of squares, which increased from site A (264) to site B (327) to site C (1163) (Table 4). The loamy sand (site B) had an intermediate variability in pool size distribution and SOC stock depletion after the bare fallow simulation (Figure 4, Table 3).

The IOM pool had the largest variability among all pools and in all soils with coefficients of variation of 48.6% (site A), 54.7% (site B) and 59.7% (site C) (Table 3). Laboratories 1, 3 and 5 measured an up to four times larger IOM pool than predicted by RothC; laboratories 3 and 5 quantified an up to seven times larger IOM pool than predicted by RothC. However, laboratories 2, 4 and 6 were able to match the IOM pool at least for sites A and B (Figure 4). Laboratory 4 isolated only half of the RothC-predicted IOM pool for site B. A systematic deviation from the RothC-predicted pool sizes was found in the RPM and DPM pools, for which all laboratories found smaller pool sizes in all three soils (Figure 4), with laboratory 6 being an exception with site C. This might be explained by the fact that laboratory 6 had a recovery rate of only 57% and that not all fractions were equally affected by C loss. On average, the quantified RPM+DPM pools together were 37% (site A), 45% (site B) and 35%

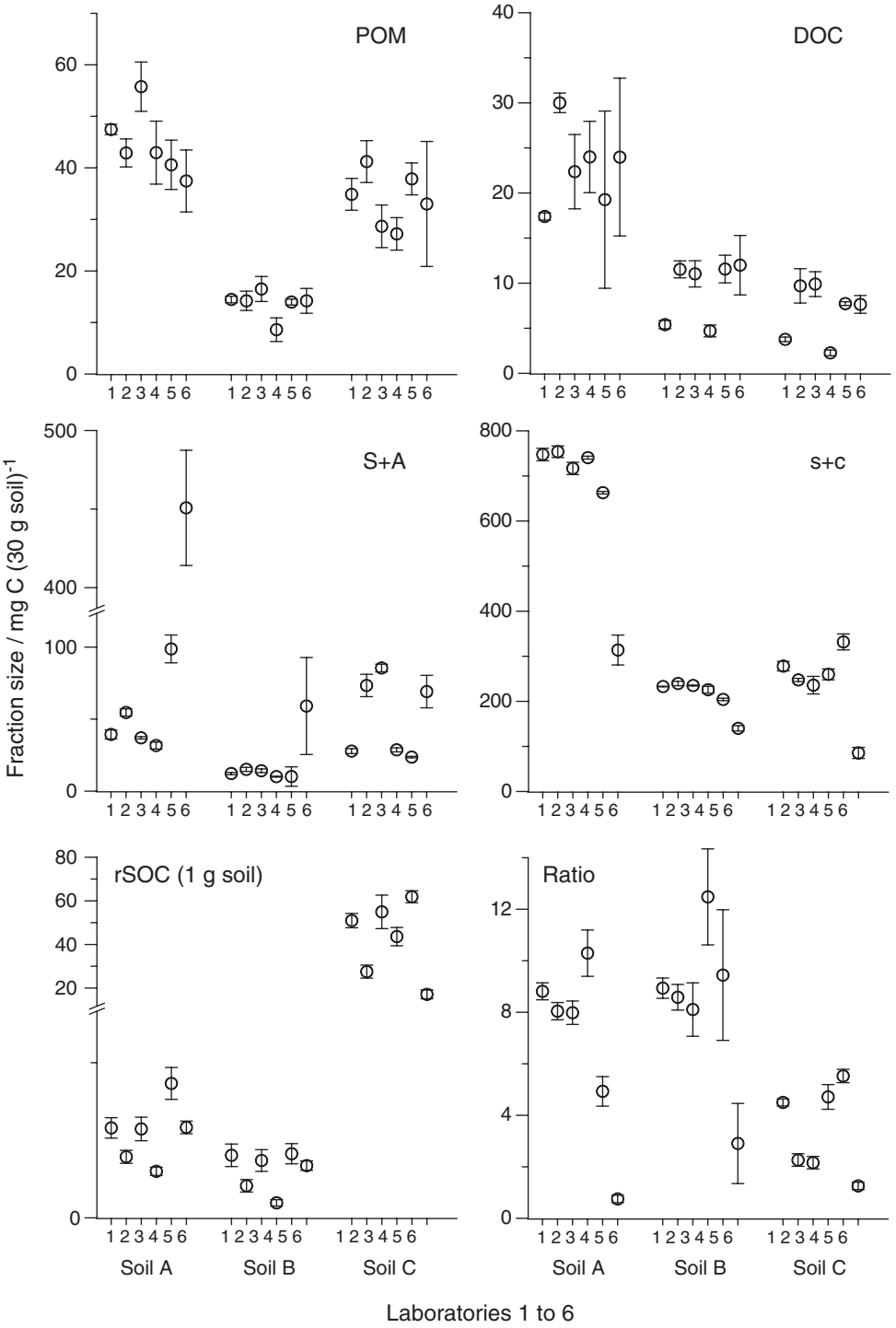

Figure 3 Isolated carbon in each measured fraction and the ratio C(<63 µm):C(>63 µm) with standard deviations for sites A, B and C and laboratories 1–6.

(site C) smaller than the RothC equilibrium pools. This indicates that insufficient POM or DOC was isolated with the existing procedure in order to match the RPM and DPM pools as predicted by RothC (Figure 4). As a result, all measured pool size distributions, with the exception of laboratory 6 for site C, led to smaller SOC losses with the simulated 40 years of bare fallow than the RothC-derived equilibrium pool size distribution. This was because the amounts of isolated POM or DOC (RPM, DPM) were too small and, the rSOC fraction was mostly too large (IOM). Laboratory 6 was able to match the RothC pools most closely but

Table 2 Coefficients of variation (%) (inter-laboratory error), residual coefficients of variation (%) (intra-laboratory error after fractionating three replicate samples) and their ratio for the fractionation results of all sites and the fractions particulate organic matter (POM), dissolved organic carbon (DOC), organic carbon in sand and stable aggregates (S+A), organic carbon in silt and clay (s+c) and resistant soil organic carbon (rSOC) plus the ratio of carbon in the fine fraction (<63 µm) and carbon in the coarse fraction (>63 µm)

	CV (inter-laboratory error)			Residual CV (intra-laboratory error)			CV: residual CV		
Fractions	A	B	C	A	B	C	A	B	C
POM	12.9	17.6	13.1	10.4	13.7	17.5	1.2	1.3	0.7
DOC	17.2	33.7	44.9	25.7	14.5	19.7	0.7	2.3	2.3
S+A	126.7	86.0	58.3	13.1	69.7	11.2	9.6	1.2	5.2
s+c	21.2	17.7	15.8	2.5	2.1	5.7	8.4	8.3	2.8
rSOC 1 g	29.1	41.5	31.1	11.0	10.8	37.6	2.6	3.8	0.8
C(<63 µm) :C(>63 µm)	48.1	29.4	53.9	7.6	18.0	8.0	6.4	1.6	6.8

Table 3 Coefficients of variations (%) among the six laboratories for each pool and site after the mean fractionation results of each laboratory have been converted into RothC pools

Pool	Site A	Site B	Site C
RPM+DPM	10.4	23.5	17.8
HUM+BIO	13.4	14.4	24.2
IOM	48.6	54.7	59.7
Total C	2.6	8.5	21.8

had a poor carbon recovery rate during the fractionation procedure and in particular for site C (64 ± 9%). Poor recovery rates are indicators of C losses during the fractionation that may lead to strong biases in the results. The mean carbon recovery rate for all laboratories was 94 ± 10% and ranged from 93.9 to 102.7% for site A, from 81.3 to 97.4% for site B and from 54.3 to 113.1% for site C.

Critical work steps

Ultrasonic dispersion. Ultrasonic dispersion and wet sieving are the first steps in the fractionation procedure and have thus a major influence on the overall SOC fraction distribution. The ultrasonic dispersion determines the amount of destroyed macroaggregates and thus the quantity of carbon washed into the <63 µm fractions. The ratio between the quantified s+c and DOC fractions (<63 µm) and the S+A and POM fraction together (>63 µm) describes the effect of ultrasonic dispersion and wet sieving. We found significant differences in C(<63 µm):C(>63 µm) between laboratories for all three soils (Figure 3), which identifies the first fractionation steps as being bias-sensitive. The amount of applied ultrasonic power (energy per unit time) varied considerably among laboratories and ranged from 22 to 90 W. In the current fractionation protocol, only the amount of energy was standardized, which allowed each laboratory to modify the amount of power and time. We found significant exponential relationships between the ratio C(<63 µm):C(>63 µm) and the amount of ultrasonic power used for all three soils ($R^2 = 0.43$, $R^2 = 0.61$ and $R^2 = 0.74$ for soils A, B and C, respectively). The <63 µm fractions increased with increasing ultrasonic power (Figure 5). This was surprising because standardization of ultrasonic dispersion in almost all SOC fractionation protocols is done by means of the applied energy with little attention being paid to the power of the ultrasonic dispenser. However, the importance of the power used is in line with the findings of Raine & So (1997), who quantified significantly more dispersion when greater power was applied but the output energy was kept constant. Aggregate breakdown during ultrasonic dispersion is mainly driven by the stresses caused by cavitation of the fluid (Mayer *et al.*, 2002). The main factor driving cavitation during ultrasonic dispersion is the acoustic pressure of the ultrasound, which depends on the density of the fluid, the sound velocity in the fluid and the sound vibration velocity amplitude (Kuttruff, 1988). As the amount of water and soil were the same in each laboratory, the acoustic pressure varied as a function of the sound vibration velocity amplitude, which is proportional to the applied power (Mayer *et al.*, 2002). We suggest that a standardized amount of power should be specified in the protocol to achieve more comparable results. As the laboratory which used the smallest amount of power achieved the best overall fit with the RothC pools (laboratory 6: 22 W), we propose that 20 W should be used. To obtain the output energy of 22 J ml^{-1}, the dispersion time would be 177 s for a volume of 161 ml (150 ml water and 30 g of soil with an assumed density of 2.65 g cm^{-3}).

Wet sieving. In the original protocol, Zimmermann *et al.* (2007) proposed that the samples should be flushed during wet sieving until the rinse water is clear. This, however, led to very different amounts of water that were used in our ring trial, ranging from 533 to 8500 ml to flush the same sample. The amount of water used was exponentially correlated with the size of the DOC fraction, with the greatest increase occurring at less than 2000 ml (Figure 6). The *a posteriori* test, which was conducted with 3000 ml of water, confirmed that the fitted exponential model is a valid predictor of the amount of DOC. When more than 2000 ml of water was used to flush the samples no significant influence of the amount of water on the amount of DOC could be observed. To exclude this source of variation, we therefore suggest using 2000 ml as a minimum amount of water to flush the sample.

Zimmermann *et al.* (2007) filtered the suspension through a 0.45-µm membrane filter to obtain the DOC fraction. We observed that filtering the suspension is, however, very time consuming, which led to the widespread use of centrifuging and decanting of the liquid before filtering. When 3000 ml of water was used for wet sieving, 10 minutes of centrifugation at 2000 ***g*** apparently sufficed to separate the solid and the liquid phase.

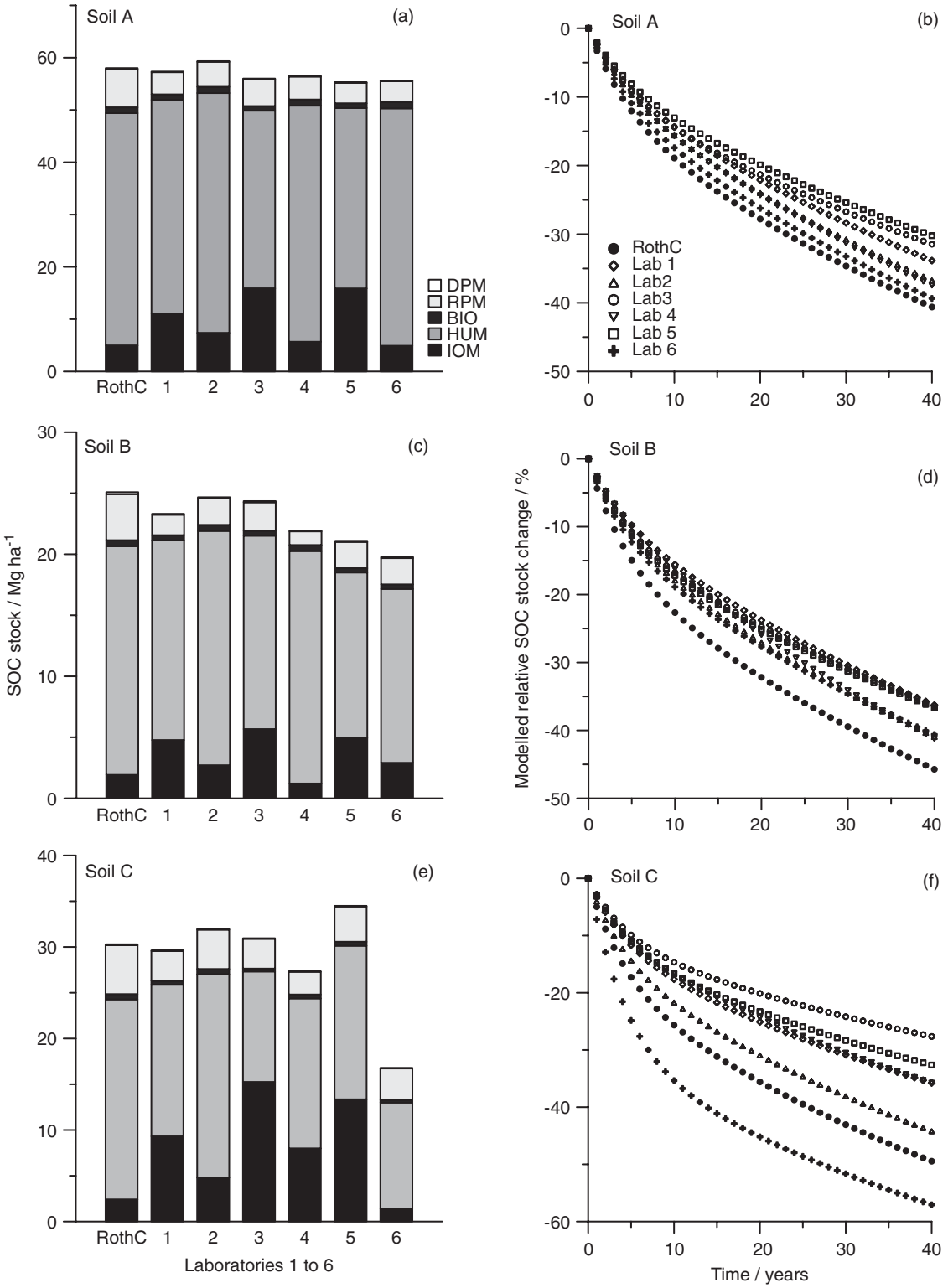

Figure 4 Modelled and measured pool sizes (a, c, e) and relative SOC stock change after using each pool size distribution to initialize the 40-year bare fallow simulation in RothC (b, d, f) for sites A, B and C.

Table 4 Deviation of each laboratory from the bare fallow simulation initialized with the RothC equilibrium pools for all three soils (% SOC change$_{\text{RothC}}$ – % SOC change$_{\text{measured pool}}$) and the residual sum of squares (RSS) for each laboratory and soil

Laboratory	Site A	Site B	Site C	RSS (laboratories)
1	−6.8	−9.4	−13.7	322
2	−3.9	−4.8	−5.2	65
3	−9.2	−9.3	−21.8	646
4	−3.1	−4.5	−13.7	218
5	−10.4	−9.1	−16.8	472
6	−1.3	−5.1	1.3	29
RSS (soils)	264	327	1163	–

Density fractionation. The comparison of modelled and laboratory-derived pool size distributions revealed that the RPM and DPM pools, which are calculated from the DOC and POM fractions, were under-estimated by all laboratories (Figure 4). This is in line with the finding of Zimmermann *et al.* (2007), who found the quantified pools to be systematically smaller than the modelled ones. They proposed that the density of SPT be increased to 2.0–2.2 g cm^{-3}; however, this is not yet in widespread practice. To our best knowledge in both published (Dondini *et al.*, 2009; Leifeld *et al.*, 2009b; Xu *et al.*, 2011; Poeplau & Don, 2013) and unpublished work, the density of 1.8 g cm^{-3} has been used. Additionally, Xu *et al.* (2011) could not confirm this systematic deviation, but found a large scatter of modelled compared with measured DPM and RPM pools. We therefore decided to maintain this density for the ring trial. However, *a posteriori* we tested whether an increased density of the SPT solution would lead to a better match of modelled and laboratory-determined RPM and DPM pools. Even with a density of 2.2 g cm^{-3} we isolated only 15% (A), 49% (B) and 20% (C) more POM than with a density of 1.8 cm^{-3}; an increase of 84% (A), 144% (B) and 69% (C) would have been needed to match the labile modelled pools (Figure 7). We therefore tested whether the amount of DOC could be increased by hot water extraction (Landgraf *et al.*, 2006) of the suspended sample before wet sieving: this increased the amount of DOC by a factor of 1.6, 1.8 and 1.9 for soils A, B and C, respectively. Summing the increased amount of POM achieved with an SPT density of 2.2 g cm^{-3} and the hot-water extracted amount of DOC, leads to a total proportion of 88% (A), 85% (B) and 90% (C) of the modelled RPM and DPM pools. It is likely that the hot water extraction would have decreased the amount of isolated POM if both steps had been conducted on the same set of samples. Therefore, we do not see a possibility of adjusting the protocol in order to match the RPM and DPM pools with POM and DOC. Moreover, Shaymukhametov *et al.* (1984) characterized the SOC in different density fractions and found that the SOC (density below 1.8–2.0 g cm^{-3}) consisted of slightly decomposed macro-organic matter of plant and animal origins with little mineral content, the SOC (density between 2.0 and 2.4 g cm^{-3}) consisted of organo-clay complexes and the heavy fraction (density < 2.4 g cm^{-3}) consisted of sand grains coated with SOC. Sollins *et al.* (2006) determined a mean residence time of 210 years for SOC separated with a density of 2.0–2.28 g cm^{-3} in a forest soil. Thus, even though an increased density does increase the amount of isolated POM and therefore comes empirically closer to the RPM and DPM pools of RothC, it is doubtful whether a fraction obtained with a density of 2.2 g cm^{-3} or greater would resemble the functional role of the labile RPM and DPM pools. We suggest that a density of 2.0 g cm^{-3} should be used and that the sample should not be hot-water extracted.

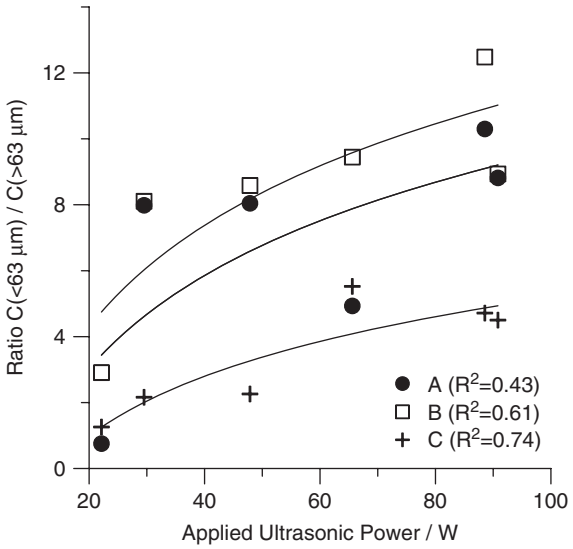

Figure 5 Ratio of C(<63 ym):C(>63 ym) as a function of applied ultrasonic power with exponential fits and R^2 values for each soil.

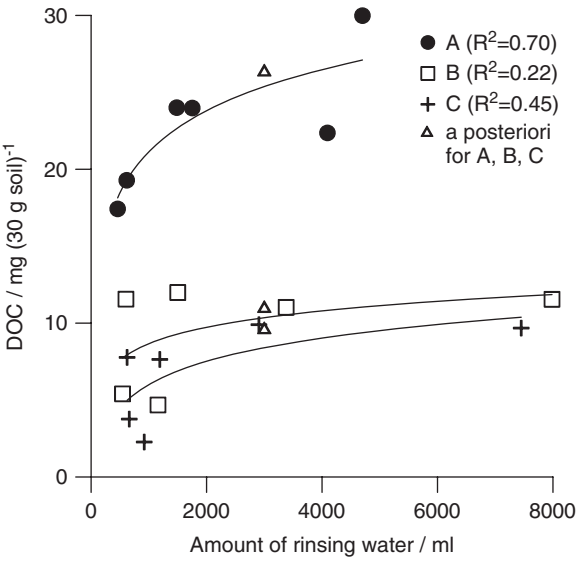

Figure 6 Mean amount of DOC extracted as a function of the amount of water used during wet sieving.

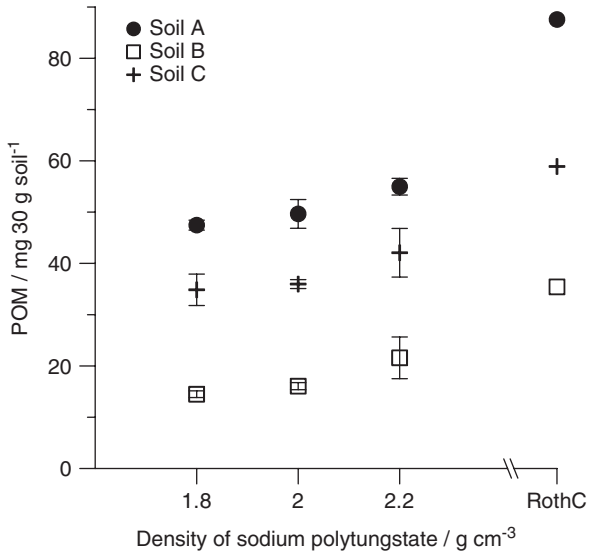

Figure 7 Size of the POM fraction obtained using different densities of sodium polytungstate compared with the fraction size, which should be obtained to match the RPM and DPM pools estimated by RothC.

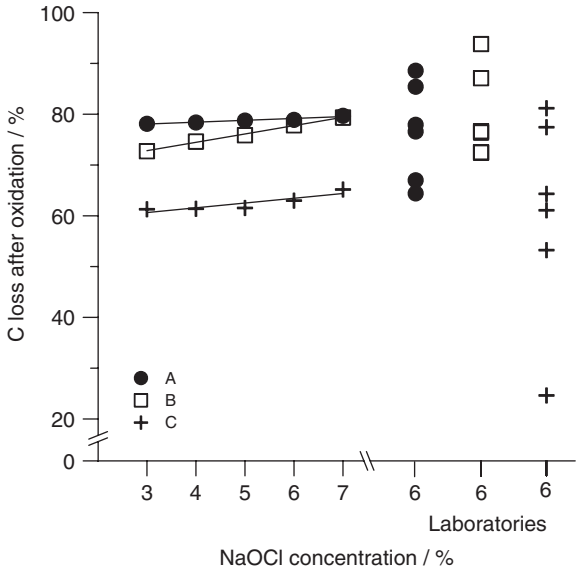

Figure 8 Carbon loss after oxidation as a function of NaOCl concentration and the results from the laboratories using a NaOCl concentration at 6% as a comparison.

NaOCl oxidation. The IOM pool had the largest variability between laboratories among all quantified pools and only three out of six laboratories were able to isolate a fraction size that was comparable to the RothC-predicted pool (Figure 4, Table 3). As chloride in the NaOCl solution is volatile, the oxidation efficiency decreases with time and is dependent on temperature during storage. We therefore recommend that fresh NaOCl solution in which the Cl concentration is known is used or that the Cl concentration is determined immediately before the solution is used for oxidation. We tested *a posteriori* whether the differences between laboratories could be explained by different concentrations of Cl in the NaOCl solution (Figure 8). All three soils had a relatively smaller sensitivity to different Cl concentrations than the variability between laboratories, indicating that differences in Cl can only partly explain the observed variability. Moreover, we found a significant positive correlation between the C(<63 µm):C(>63 µm) ratio and the proportion of oxidizable carbon in the s+c fraction ($r = 0.43$, data not shown). This indicates that quality and quantity of SOC in the s+c fraction is again determined by ultrasonic dispersion through the breakdown of macroaggregates and thus the release of occluded carbon. The better the dispersion efficiency of the ultrasonic dispersion, the more potentially labile SOC is found in the s+c fraction and thus the more oxidizing agent is needed to isolate the same amount of rSOC. This hypothesis is in line with the findings of Siregar *et al.* (2005), who treated 12 different soils with NaOCl and determined an oxidation efficiency range from 12 to 72%, which was negatively correlated with the clay content of the soils ($r = 0.45$). Similarly, Kleber *et al.* (2005) explain the same results with either a varying degree of recalcitrance of SOM or a different degree of protection exerted by the soil matrix. Thus, the oxidation efficiency is strongly dependent on the quality of the organic matter present. Furthermore, the differences among laboratories in the size of the s+c fraction, which is again determined by ultrasonic dispersion, potentially contribute to explaining the variability in the size of the rSOC fraction. The oxidation is conducted with 1 g of the sample but the fraction size is determined by multiplying the resistant C in 1 g with the total amount (g) of s+c. The variability of the total amount of s+c material thus contributed about one-third of the coefficient of variation for the rSOC fraction. This stresses the need for an improved standardized ultrasonic procedure. Another possible source of the large laboratory bias could be the different intensities of shaking the sample after the addition of fresh NaOCl. To ensure a complete oxidation of the oxidizable C in the soil sample, the sedimented soil *a posteriori* in the vessel must be brought fully in suspension; ideally, a vortex mixer should be used. We did not test this in the current study.

Table 5 Mean deviations of the quantified POM+DOC and POM+DOC+S+A fractions from the RothC equilibrium RPM+DPM pools (Mg C ha^{-1}) for all three soils

	A		B		C	
Laboratory	POM+DOC	POM+DOC+S+A	POM+DOC	POM+DOC+S+A	POM+DOC	POM+DOC+S+A
1	−2.98	−0.35	−2.05	0.32	−2.04	−0.97
2	−2.44	1.20	−1.01	5.24	−1.53	−0.21
3	−2.09	0.38	−2.06	5.22	−1.37	−0.14
4	−2.83	−0.72	−2.83	−0.39	−2.61	−1.73
5	−3.31	3.28	−1.45	0.56	−1.55	−0.67
6	−3.20	27.00	−1.94	5.50	−1.68	2.00

The role of the S+A fraction. The SOC in the S+A fraction is probably the most heterogeneous SOC fraction in terms of composition and stability. It comprises POM >0.63 µm, which is visually detectable, occluded POM in aggregates with different cohesive strength, and also SOC in clay-sized organo-mineral particles within these aggregates and as coatings on sand grains (which might have a long turnover time) (Shaymukhametov *et al.*, 1984). This, however, contradicts the perception that SOC fractions should ideally be unique and non-composite in order to match model pools (Smith *et al.*, 2002). Zimmermann *et al.* (2007) considered the S+A fraction to be physically stabilized and thus slower cycling than the labile fractions of DOC and POM. However, recent work has shown that the S+A fraction had a larger or only slightly smaller sensitivity to land-use change than DOC (Poeplau & Don, 2013). Dondini *et al.* (2009) observed a larger proportion of C_4-derived carbon in the S+A fraction than in the POM fraction 14 years after a C_3-C_4 vegetation change. This indicates that this fraction would contain a larger proportion of 'fresh' SOC than of stabilized SOC. In contrast, Leifeld & Fuhrer (2009) calculated mean residence times (MRTs) of >100 years for the S+A fraction, while the POM fraction was not older than 5 and 7 years in the upper 4 cm of a subalpine pasture and meadow, respectively. This, however, does not exclude the possibility that the S+A fraction consists of old SOC, but also a substantial amount of fresh SOC. We therefore calculated the RPM and DPM pools by adding S+A to the POM and DOC fractions to test this. Thirteen of 18 recalculated measurements (six laboratories and three soils) achieved a better match with the RPM and DPM pools than with the proposed calculation with only POM and DOC (Table 5). Eight of these 13 recalculated measurements with POM, DOC and S+A were still less than the RPM and DPM pools from RothC. Laboratory 6, which used the smallest amount of ultrasonic power and thus isolated the largest S+A fraction, would over-estimate the RPM+DPM fractions when S+A is included. In contrast, laboratory 1, which used the greatest amount of ultrasonic power, would achieve a better match with the RPM+DPM pools for all three soils if the S+A fraction was included (Table 5). To maintain the current concept of the fractionation method, we therefore suggest that a small amount of ultrasonic power (20 W) is used. However, to identify the functional role of the S+A fraction, as well as that of all other fractions, the mean residence time (MRT) of each fraction should be investigated by using repeated ^{14}C measurements (Baisden *et al.*, 2011). Only this information would permit us to validate a mechanistic framework for the existing fractionation procedure, which is currently based only on conceptual considerations and empirical relations of measured fraction sizes and the modelled pools. Even so, a perfect fit of fractions and conceptual pools will not be achievable because the nature of SOC is a continuum rather than consisting of distinct pools. In the specific case of RothC this is especially true for the IOM pool, which is estimated using the Falloon equation (Falloon *et al.*, 1998), while the actual size of IOM in a specific soil or even the existence of such a pool remains very uncertain. Fractionation, as well as modelling with different functional pools, will thus remain an approximation.

Conclusions

The conducted ring trial revealed that the fraction procedure described by Zimmermann *et al.* (2007) was not described precisely enough and led to individual laboratory-specific adjustments and thus significantly different results for three different soils and all fractions. The amount of applied ultrasonic power during ultrasonic dispersion as the first step of the fractionation scheme strongly drives the disruption of aggregates and thus influences the quality and size of all fractions. We thus suggest standardizing not only the ultrasonic energy, but also the ultrasonic power (20 W). The amount of isolated DOC is a function of the amount of water used for wet sieving, which demands a minimum amount of water (2000 ml) to be used. Calculating the distribution of SOC over pools of the RothC biogeochemical model led systematically to a stronger allocation of SOC to stable model pools than with the RothC model's equilibrium pool size distributions. Neither increasing the density of the SPT solution for density fractionation, nor increasing the concentration of NaOCl for isolating the rSOC fraction, could solve this entirely. Moreover, a substantial part of the S+A fraction might be young and fast cycling material, contrary to the original concept that this fraction coincides with a relatively stable SOM pool. This hypothesis needs to be further tested and possibly an additional fractionation step would be required to split the S+A fraction into a young component, which adds to the RPM and DPM pools in the RothC model, and an old part, which would actually relate to the turnover of the HUM pool. However, to achieve more comparable results between laboratories and to achieve a better fit with RothC-predicted pools by maintaining the current conceptual framework of this soil carbon fractionation method, we suggest an improved fraction protocol (see File S1).

Supporting Information

The following supporting information is available in the online version of this article:

File S1. Improved fractionation protocol.

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References

Amelung, W. & Zech, W. 1999. Minimisation of organic matter disruption during particle-size fractionation of grassland epipedons. *Geoderma*, **92**, 73–85.

Baisden, W.T., Parfitt, R., Ross, C., Schipper, L. & Canessa, S. 2011. Evaluating 50 years of time-series soil radiocarbon data: towards routine calculation of robust C residence times. *Biogeochemistry*, **9**, 1–9.

Blair, G.J., Lefroy, R.D.B. & Lise, L. 1995. Soil carbon fractions based on their degree of oxidation and the development of a carbon management indey for agricultural systems. *Australian Journal of Agricultural Research*, **46**, 1459–1466.

Bol, R., Poirier, N., Balesdent, J. & Gleixner, G. 2009. Molecular turnover time of soil organic matter in particle-size fractions of an arable soil. *Rapid Communications in Mass Spectrometry*, **23**, 2551–2558.

Christensen, B.T. 2001. Physical fractionation of soil and structural and functional complexity in organic matter turnover. *European Journal of Soil Science*, **52**, 345–353.

Conant, R.T., Six, J. & Paustian, K. 2004. Land use effects on soil carbon fractions in the southeastern United States. II. Changes in soil carbon fractions along a forest to pasture chronosequence. *Biology & Fertility of Soils*, **40**, 194–200.

Dondini, M., Hastings, A., Saiz, G., Jones, M.B. & Smith, P. 2009. The potential of Miscanthus to sequester carbon in soils: comparing field measurements in Carlow, Ireland to model predictions. *GCB Bioenergy*, **1**, 413–425.

Elliott, E.T., Paustian, K. & Frey, S.D. 1996. Modelling the measurable or measuring the modelable: a hierarchical approach to isolating meaningful soil organic matter fractions. In: *Evaluation of Soil Organic Matter Models Using Existing Long-term Datasets* (eds D.S. Powlson, P. Smith & J.U. Smith), pp. 162–179. Springer-Verlag, Berlin.

Falloon, P., Smith, P., Coleman, K. & Marshall, S. 1998. Estimating the size of the inert organic matter pool from total soil organic carbon content for use in the Rothamsted carbon model. *Soil Biology & Biochemistry*, **30**, 1207–1211.

Helfrich, M., Flessa, H., Mikutta, R., Dreves, A. & Ludwig, B. 2007. Comparison of chemical fractionation methods for isolating stable soil organic carbon pools. *European Journal of Soil Science*, **58**, 1316–1329.

Jenkinson, D.S. & Rayner, J.H. 1977. The turnover of soil organic matter in some of the Rothamsted classical experiments. *Soil Science*, **123**, 298–305.

Kleber, M., Mikutta, R., Torn, S. & Jahn, R. 2005. Poorly crystalline mineral phases protect organic matter in acid subsoil horizons. *European Journal of Soil Science*, **56**, 717–725.

Krull, E.S. & Skjemstad, J.O. 2003. delta(13)C and delta(15)N profiles in (14)C-dated Oxisol and Vertisols as a function of soil chemistry and mineralogy. *Geoderma*, **112**, 1–29.

Kuttruff, H. 1988. *Physik und Technik des Ultraschalls*. S. Hirzel Verlag, Stuttgart.

Landgraf, D., Leinweber, P. & Makeschin, F. 2006. Cold and hot water-extractable organic matter as indicators of litter decomposition in forest soils. *Journal of Plant Nutrition and Soil Science*, **169**, 76–82.

Leifeld, J. & Kogel-Knabner, I. 2001. Organic carbon and nitrogen in fine soil fractions after treatment with hydrogen peroxide. *Soil Biology & Biochemistry*, **33**, 2155–2158.

Leifeld, J. & Fuhrer, J. 2009. Long-term management effects on soil organic matter in two cold, high-elevation grasslands: clues from fractionation and radiocarbon dating. *European Journal of Soil Science*, **60**, 230–239.

Leifeld, J., Zimmermann, M., Fuhrer, J. & Conen, F. 2009a. Storage and turnover of carbon in grassland soils along an elevation gradient in the Swiss Alps. *Global Change Biology*, **15**, 668–679.

Leifeld, J., Reiser, R. & Oberholzer, H.R. 2009b. Consequences of conventional versus organic farming on soil carbon: results from a 27-year field experiment. *Agronomy Journal*, **101**, 1204–1218.

Mayer, H., Mentler, A., Papakyriacou, M., Rampazzo, N., Marxer, Y. & Blum, W.E.H. 2002. Influence of vibration amplitude on the ultrasonic dispersion of soils. *International Agrophysics*, **16**, 53–60.

Motavalli, P.P., Palm, C.A., Parton, W.J., Elliott, E.T. & Frey, S.D. 1994. Comparison of laboratory and modeling simulation methods for estimating soil carbon pools in tropical forest soils. *Soil Biology & Biochemistry*, **26**, 934–944.

Paustian, K. 2001. Modelling soil organic matter dynamics- global challenges. In: *Management of Soil Organic Matter* (eds R.M. Rees, B.C. Ball, C.D. Campbell & C.A. Watson), pp. 43–53. CAB International, Wallingford.

Poeplau, C. & Don, A. 2013. Sensitivity of soil organic carbon stocks and fractions to different land-use changes across Europe. *Geoderma*, **192**, 189–201.

Raine, S.R. & So, H.B. 1997. An investigation of the relationships between dispersion, power, and mechanical energy using the end-over-end shaking and ultrasonic methods of aggregate stability assessment. *Australian Journal of Soil Research*, **35**, 41–53.

Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., *et al.* 2011. Persistence of soil organic matter as an ecosystem property. *Nature*, **478**, 49–56.

Shaymukhametov, M.S., Titova, N.A., Travnikova, L.S. & Labenets, Y.M. 1984. Use of physical fractionation methods to characterize soil organic matter. *Soviet Soil Science*, **16**, 117–128.

Siregar, A., Kleber, M., Mikutta, R. & Jahn, R. 2005. Sodium hypochlorite oxidation reduces soil organic matter concentrations without affecting inorganic soil constituents. *European Journal of Soil Science*, **56**, 481–490.

Six, J., Guggenberger, G., Paustian, K., Haumaier, L., Elliott, E.T. & Zech, W. 2001. Sources and composition of soil organic matter fractions between and within soil aggregates. *European Journal of Soil Science*, **52**, 607–618.

Smith, J.U., Smith, P., Monaghan, R. & Macdonald, J. 2002. When is a measured soil organic matter fraction equivalent to a model pool? *European Journal of Soil Science*, **53**, 405–416.

Sollins, P., Swanston, C., Kleber, M., Filley, T., Kramer, M., Crow, S., *et al.* 2006. Organic C and N stabilization in a forest soil: evidence from sequential density fractionation. *Soil Biology & Biochemistry*, **38**, 3313–3324.

Trumbore, S.E., Vogel, J.S. & Southon, J.R. 1989. AMS C-14 measurements of fractionated soil organic matter- an approach to deciphering the soil carbon cycle. *Radiocarbon*, **31**, 644–654.

Xu, X.L., Liu, W. & Kiely, G. 2011. Modeling the change in soil organic carbon of grassland in response to climate change: effects of measured versus modelled carbon pools for initializing the Rothamsted Carbon model. *Agriculture, Ecosystems & Environment*, **140**, 372–381.

Zimmermann, M., Leifeld, J., Schmidt, M.W.I., Smith, P. & Fuhrer, J. 2007. Measured soil organic matter fractions can be related to pools in the RothC model. *European Journal of Soil Science*, **58**, 658–667.