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Technical note: The recovery rate of free particulate organic matter is strongly reduced by conventional density fractionation of soil samples

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Abstract. Ultrasonication combined with density fractionation (USD) is a method widely used to quantify soil organic matter pools. A selective fractionation of free particulate organic matter (fPOM) is crucial to avoid co-extraction of retained fPOM along with occluded particulate organic matter (oPOM). In the present work, artificial fPOM was extracted from two mineral matrices, sandy and loamy, after applying different approaches for merging sample and dense medium. It could be shown that pouring the dense solution to the mineral matrices leads to low recovery, whereas trickling the sample into the solution, rotating after fill-up or applying a minimal and defined amount of ultrasound to swirl up the sample causes nearly full recovery of the artificial fPOM. Applied to natural soils, the results confirmed the low extraction rate of the fill-up approach. Moreover, it was possible to show that the rotational approach results in only a slightly increased extraction rate, whereas the ultrasound approach leads to a release of oPOM into the fPOM fraction due to disruption of soil macro-aggregates. The trickle approach appears to be the most appropriate way among the tested methods to achieve complete and selective extraction of fPOM from natural soil samples.

Introduction

In soils, particulate organic matter (POM) occurs free (fPOM) as well as occluded within soil aggregates (oPOM) (Golchin et al., 1994). Both organic matter pools with different chemical composition, structure and decomposition rates are task of widespread experimental issues regarding carbon pool balances, soil structural stability or turnover times (von Lützow et al., 2007; Wagai et al., 2009; Büks and Kaupenjohann, 2016; Graf-Rosenfellner et al., 2016). A widely used method to separate fPOM and oPOM is ultrasonication combined with density-fractionation (USD) (Kaiser and Berhe, 2014). Both POM fractions are thereby determined indirectly by quantification of the operational non-aggregated particulate free light fraction (fLF) and the occluded light fraction within soil aggregates (oLF) (Golchin et al., 1994; Büks and Kaupenjohann, 2016). The congruence between light fractions and actual POM pools is reduced by low recovery rates and the carryover between the pools as recently shown for oPOM and mineral-associated organic matter (MOM) (Büks et al., 2021). A sharp separation without cross-contamination between the measured pools is therefore necessary.

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This work focused on the separation of fPOM and oPOM, driven by two observations: A pre-experiment following the specifications given below for the extraction of POM from soil samples showed a separation of 28.7±3.1 mg fPOM when the density fractionation solution was added to the soil sample, but 44.8±7.4 mg when the sample was gently trickled through the solution (n=3, t-test, $p<0.05$). The first of the two approaches is the original and now commonly used in soil science (e.g. Golchin et al., 1994; Cerli et al., 2012; Schrumpf et al., 2013). However, many works applying the USD do not specify the method of bringing soil sample and dense solution together. Due to the very different performance of both approaches shown, the aim of this work was to compare methods in order to identify such with most accurate separation of fPOM and oPOM.

Material and methods

The simple scenario: Extraction of LD-PE particles from mineral matrices

In a first experiment, two simple model soils were prepared from a mineral matrix of calcinated fine sand (89.7 % sand, 9.3 % silt, 1.0 % clay) and a calcinated clayey silt (8.7 % sand, 69.7 % silt, 21.6 % clay), each amended with 1 wt% of weathered low-density polyethylene made from cryo-milled film (LD-PE, weathered 96 h at 1000 W m^{-2}, 38°C and 50 % RH following DIN EN ISO 4892-2/3, $x_{10\%}$=246 µm, $x_{50\%}$=435 µm, $x_{90\%}$=691 µm, ρ=0.92 g cm^{-3}) as a well-defined fPOM representative. This setting provides low physicochemical interaction between mineral and organic particles, that allowed for focusing on artifacts caused by mechanical reasons such as sedimentation behavior and impeded flotation. The textures of the two mineral matrices represent different sedimentation rates, likely affecting the recovery rate of the LD-PE.

Four scenarios with each six replicates of 20 g soil sample and 100 ml 1.6 g cm^{-3} dense sodium polytungstate solution (SPT) in 200 ml centrifuge bottles were tested: One in which the soil samples were gently *filled up* with solution, one in which the soil samples were *trickled* into the solution, one in which the flasks were gently *rotated* horizontally 3x with 20 rpm to unhitch the sedimented soil matrix from the bottom of the flask, and one that was agitated by ultrasonication (Branson© Sonifier 250, sonotrode diameter 13 mm, frequency 40 kHz, immersion depth 15 mm, power output 52.06±1.67 J s^{-1}) until the sediment was completely swirled up (*pre-sonicated*). The respective time of sonication (t_{min}) was determined to be 7.0±1.3 sec for the sandy and 34.0±1.9 sec for the loamy soil (*see Supplements*). The corresponding energy densities w_{min} were calculated following North (1976) and amounted to 3.0±0.5 J ml^{-1} and 14.7±0.8 J ml^{-1}, respectively.

In order to extract the POM, samples were centrifuged at 3,500 G for 26 min. The floated LD-PE was collected by use of a water-jet vacuum pump and cleaned with deionized water to remove remaining SPT salt by use of a 0.45 µm cellulose acetate membrane-filter until the electrical conductivity of the filtrate fell below 50 µS cm^{-1}. The extracted LD-PE was then flushed off the filter with deionized water into aluminum bottles, frozen at -20°C, lyophilized and finally weighed to determine the recovery rate.

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The complex scenario: Extraction of POM from natural soils

In a second experiment, two topsoil samples, sandy (89.7 % sand, 9.3 % silt, 1.0 % clay) and loamy (25.5 % sand, 55.9 % silt, 18.7 % clay), were air-dried and sieved to receive aggregates of 250 to 2000 µm in diameter. In six-fold replication, 20 g of soil aggregates were gently adjusted via spray to a water content of 200 mg g^{-1} dry soil, low enough to avoid aggregates sticking to each other or to the flask, and incubated for 2 weeks at 20 °C in the dark. After the removal of shoots, soil samples and SPT solution were merged following the four approaches and the fPOM was extracted in the same manner given above. Subsequently, the samples were refilled to 100 ml of SPT per flask and treated by application of w=50 J ml^{-1} in the *fill-up*, *trickle* and *rotate* scenarios as well as w=50 J ml^{-1}-w_{min} to the *pre-sonicated* variant. Afterwards the oPOM was extracted as above, followed by centrifugation, collection, cleaning, freezing, lyophilization and quantification by weighing. Finally, all POM samples were ground, dried at 105°C and the amount of organic carbon was determined using an Elementar Vario EL III CNS Analyzer.

Results

Recovery rates from mineral matrices

The results show that *fill-up*, the commonly used method, provided by far the lowest recovery rate in both the sandy and clayey mineral matrix (68.3±9.0 % and 58.9±13.7 % of the applied LD-PE, respectively). In contrast, *trickle*, *rotate* and *pre-sonicated* have similarly high recovery rates ranging from 90.4±5.8 % to 98.2±1.1 % across all samples (Fig. 1).

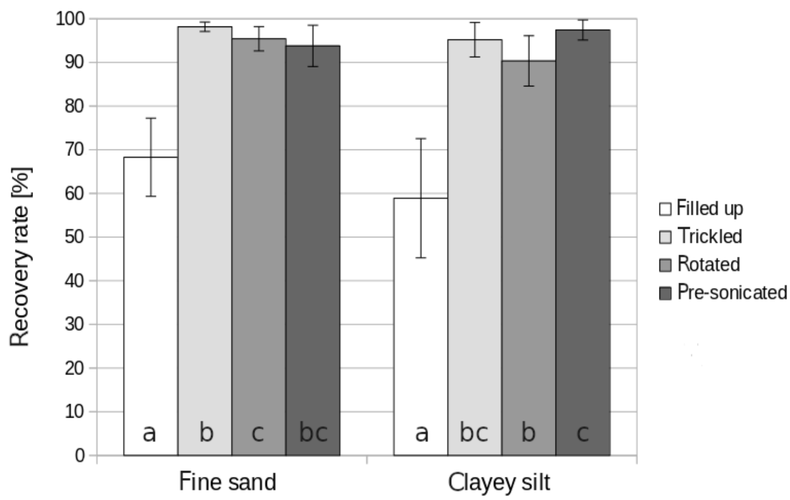

Figure 1: Recovery rates of fPOM (weathered LD-PE) from mineral matrices after fractionation with 1.6 g cm^{-3} dense SPT solution using different approaches (n=6, t-test, p<0.05). Small letters indicate Tukey's characters.

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Recovery rate and POM quality in natural soil samples

The application of all four approaches to aggregates of the loamy natural soil showed, that the *filled up* samples released by far the lowest amount of fPOM and percentage of total SOC, followed by the *rotated* and clearly excelled several times over by the *trickled* and *pre-sonicated* variant (Table 1). Unlike the other fPOM, the *pre-sonicated* fraction contained large amounts of dark fine material in addition to coarse POM. This comes along with the lowest C:N ratio, slightly reduced compared to the other fPOMs, and an increased C:N ratio in the residuum. The yield of the *pre-sonicated* oPOM fraction was strongly reduced compared to the other variants and showed the release of almost exclusively fine material. This is in contrast to *fill-up*, *trickle* and *rotate*, which had similar appearance with traces of coarse material. In sum, the *trickled* sample had the largest release of ΣPOM=fPOM+oPOM, followed by the *rotated* samples.

Tab. 1: Soil organic matter (SOM) release of a loamy topsoil after different approaches for merging sample and dense medium. fPOM refers to the free particulate organic matter floating after application of 0 J ml^{-1}, oPOM to the occluded particulate organic matter released after application of 50 J ml^{-1} (*in case of the variant with minimum ultrasonication 15 and 35 J ml^{-1}, respectively). C_{tot} refers to the total carbon content of each organic matter fraction including the residuum. ± refers to the standard deviation. Small superscripts are Tukey's characters ($p<0.05$).

Loamy soil	filled up	trickled	rotated	pre-sonicated*
fPOM				
oPOM				
fPOM (g kg^{-1} dry soil)	5.44±1.67 ^{a}	14.94±1.96 ^{b}	9.68±0.95 ^{c}	15.64±1.69 ^{b}
oPOM (g kg^{-1} dry soil)	13.42±1.43 ^{a}	12.39±2.19 ^{a}	12.82±0.87 ^{a}	1.96±1.67 ^{b}
ΣPOM (g kg^{-1} dry soil)	18.86±3.10 ^{a}	27.33±4.15 ^{b}	22.20±1.82 ^{c}	17.60±3.36 ^{a}
fPOM (% C_{tot})	5.18±1.46 ^{a}	13.78±3.01 ^{b}	8.62±0.88 ^{c}	17.13±1.16 ^{d}
oPOM (% C_{tot})	17.31±5.00 ^{a}	13.54±1.21 ^{a}	13.88±0.83 ^{a}	1.86±1.65 ^{b}
residuum (% C_{tot})	77.50±5.76 ^{abc}	72.68±2.20 ^{a}	77.50±0.76 ^{b}	81.01±1.16 ^{c}
fPOM (C:N ratio)	26.05±0.93 ^{ab}	25.34±1.55 ^{ac}	27.62±1.55 ^{b}	24.15±0.61 ^{c}
oPOM (C:N ratio)	22.00±0.89 ^{a}	20.07±0.29 ^{b}	20.52±0.78 ^{b}	20.23±5.45 ^{ab}
residuum (C:N ratio)	12.15±0.27 ^{a}	11.79±0.32 ^{a}	12.01±0.35 ^{a}	12.53±0.20b ^{b}

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Similar to the loamy soil, the *filled up* sandy soil samples showed the smallest amount of extracted fPOM followed by the *rotated* ones (Table 2). The *pre-sonicated* and *trickled* samples released the highest amount of fPOM significantly increased by about 93 % compared to the *filled up* samples. This pattern appears similarly with SOC. The release of oPOM from *pre-sonicated* samples was reduced compared to the *filled up*, *trickled* and *rotated* samples. In sum, the *filled up* samples released the smallest and the *trickled* sample the highest amount of ΣPOM.

In contrast to the rougher treated loamy samples (15 J ml^{-1}), *pre-sonication* of sandy samples with 3 J ml^{-1} did not cause any additional release of fine material within the fPOM fraction. There were no significant differences of the C:N ratio between all variants, and all fPOM fractions showed a very similar appearance. On the other hand, the oPOM fractions of the *filled up* samples and, to a lesser extent, the *rotated* samples showed an increased number of coarse particles similar to those found within the fPOM fraction, whereas the *pre-sonicated* oPOM fraction contained nearly no coarse material. This comes along with the occurrence of the highest oPOM C:N ratio in the *filled up* samples and the lowest in the *pre-sonicated* and *trickled* samples. Similar to the loamy samples, the residual C:N ratios in all sandy soil variants are low compared to the fPOM and oPOM fractions, and showed the highest values in the *filled up* and *rotated* variants.

Tab. 2: Soil organic matter (SOM) release of a sandy topsoil after different approaches for merging sample and dense medium. fPOM refers to the free particulate organic matter floating after application of 0 J ml^{-1}, oPOM to the occluded particulate organic matter released after application of 50 J ml^{-1} (*in case of the variant with minimum ultrasonication 3 and 47 J ml^{-1}, respectively). C_{tot} refers to the total carbon content of each organic matter fraction including the residuum. ± refers to the standard deviation. Small superscripts are Tukey's characters ($p<0.05$).

Sandy soil	fill-up	trickle	rotate	US*
fPOM				
oPOM				
fPOM (g kg^{-1} dry soil)	6.86±1.37 ^{a}	13.52±2.97 ^{b}	9.37±1.79 ^{c}	12.97±2.81 ^{b}
oPOM (g kg^{-1} dry soil)	8.84±0.20 ^{a}	7.28±2.12 ^{ab}	7.81±1.65 ^{a}	5.73±1.33 ^{b}
ΣPOM (g kg^{-1} dry soil)	15.70±1.57 ^{a}	20.80±5.09 ^{b}	17.18±3.44 ^{a}	18.70±4.14 ^{ab}
fPOM (% C_{tot})	4.68±0.91 ^{a}	8.97±1.62 ^{b}	6.67±1.36 ^{c}	11.46±2.16 ^{d}
oPOM (% C_{tot})	8.23±1.67 ^{a}	6.37±2.10 ^{ab}	7.65±1.69 ^{a}	4.75±1.39 ^{b}

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residuum (% C_{tot})	87.10±2.26 [a]	84.66±2.33 [ab]	85.68±1.16 [ab]	68.79±2.84 [b]
fPOM (C:N ratio)	20.84±1.35 [a]	19.46±0.96 [a]	19.88±1.01 [a]	20.81±1.87 [a]
oPOM (C:N ratio)	18.94±0.47 [a]	16.02±0.66 [b]	17.39±1.09 [c]	15.45±0.77 [b]
residuum (C:N ratio)	8.76±0.21 [a]	9.40±0.48 [b]	8.75±0.15 [a]	9.13±0.52 [ab]

Discussion

It was possible to show significant differences in the extraction performance of the different approaches. As demonstrated in the first experiment, the recovery rate of LD-PE particles from sandy and loamy mineral matrices is strongly reduced by use of the conventional *fill-up* method. This implies that filling the dense solution on top the soil sample causes parts of the fPOM to be buried under the mineral matrix. Consequently, it is suggested that the *fill-up* approach is not an adequate method to avoid incomplete extraction of fPOM. The retained fPOM will be in turn found within the oPOM fraction leading to both underestimation of the fPOM and overestimation of the oPOM fraction. The other approaches were shown to have similar extraction performance in terms of non-occluded, weakly interacting LD-PE particles within a solely mineral matrix.

However, during extraction of POM natural soils provide additional interference between SOM and the mineral phase such as by physiochemical interaction of surfaces, biofilm formation, density gradients of organic matter as well as occlusion within soil aggregates. The second experiment was therefore performed with samples of aggregates from sandy and loamy natural soils.

Similar to the first experiment, in both the sandy and loamy soil the extracted amount of fPOM was strongly reduced in the *filled up* variant, but also in the *rotated* variant, compared to the two others. Since the fPOM of the sandy soil shows a similar C:N ratio and composition of coarse particles across all approaches, the fPOM of all sandy soil variants can be considered free of (fine particulate) oPOM. In turn, the oPOM fractions of the *filled up* and *rotated* variant contain more coarse material and have a significantly higher C:N ratio compared to the others. In consequence, the *trickling* and *pre-sonication* caused less cross-contamination and are, thus, both considered yielding and sharp methods to extract fPOM from sandy soil samples. Due to its higher total POM yield, *trickling* is to be preferred over *pre-sonication* for the quantification of soil carbon pools.

In contrast to the sandy soil, the fPOM of *pre-sonicated* loamy sample contains significant amounts of fine material and a decreased C:N ratio. This artifact can be explained by the application of mechanical stress through the use of w_{min} to swirl up the soil sample. The ultrasound led to the disruption of macro-aggregates and the release of a more strongly degraded soil organic matter fraction. As shown by Wagai et al. (2009) and Cerli et al. (2012), such fractions can have in some cases a lower C:N ratio. The effect is missing in the sandy soil samples, which were treated with only 3 J ml^{-1}, but appears at 15 J ml^{-1} with loamy soils. Following Kaiser and Berhe (2014), the applied energy is well below ultrasonic levels that have been reported to disperse soil aggregates, but may still break down very

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weak macro-aggregates. In contrast, data of North (1979) and Golchin et al. (1994) point out, that even low dispersive energies <10 J g^{-1} already lead to a strong release of clay particles from aggregates of a clayey soil.

In addition, the oPOM yield of the *pre-sonicated* variant is strongly reduced coming along with an increased SOC content of the residuum. This effect did not appear with plastic particles in the first experiment and might be related to ultrasonic comminution of POM as described in Büks et al. (2021). Although *pre-sonication* provides the highest fPOM yield in loamy soils, this method is not recommended due to the low total POM yield as well as aggregate disruption and cross-contamination between POM pools. The greatest release of total POM by far is achieved using the *trickle* approach, which caused no signs of cross contamination.

Based on the performance of the four approaches (Table 3), the following general recommendations are made on their use. The commonly used *fill-up* method is greatly affected by its very low fPOM recovery and fPOM artifacts within the oPOM fraction. *Rotating* shows characteristics similar to the *fill-up* approach. It allows a higher, but still insufficient POM recovery from natural soil samples, while applying an undefined amount of mechanical stress to aggregates. Together with the *trickle* approach, *pre-sonication* shows the highest fPOM yield, might be effective when applied to sandy soils, but causes cross-contamination and low oPOM yield with loamy soils. The *trickling* method, in turn, avoids mechanical agitation, has high recovery of fPOM combined with the highest total POM yield and hardly shows any visible nor measurable cross-contamination. Suitable for a wide range of water contents, it might be, however, inadequate for the application on very moist or saturated field-fresh or pre-incubated samples that adhere to the sampling container in such way that it is difficult to transfer without mechanical stress e.g. by use of a spoon.

Table 3: Performance of the four different approaches (fill-up, trickling, rotation and pre-sonication). oPOM recovery is called unknown, if the the oPOM fraction is contaminated with fPOM material.

		recovery		**cross-contamination**	
		fPOM	**oPOM**	**oPOM in fPOM**	**fPOM in oPOM**
sandy	**filled up**	low	unknown	no	yes
	trickled	high	high	no	n
	rotated	medium	unknown	no	yes
	pre-sonicated	high	low	no	n
loamy	**filled up**	low	unknown	no	yes
	trickled	high	high	no	no
	rotated	medium	unknown	no	yes
	pre-sonicated	high	low	yes	no

Based on the findings, a modification of the traditional approach is recommended, that includes gentle *trickling* of field fresh or pre-incubated samples with water contents below

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field capacity into the density separation solution instead of adding the solution to the sample. This avoids burying significant parts of the fPOM under the mineral phase during the extraction of the fLF, which is then co-extracted along with the oPOM in the following step.

Conclusion

The complete and selective extraction of POM fractions with ultrasonication/density fractionation (USD) is an important step of SOM pool quantification. It is shown, that the common approach (filling dense solution onto the soil sample) causes strongly decreased recovery of fPOM and a cross-contamination with fPOM and oPOM between the free and occluded light fractions. This causes the misquantification of both fractions and might lead to the underestimation of the labile and an overestimation of the intermediate soil carbon pool. In addition to a number of unsuitable alternatives, *trickling* (the soil sample into the dense solution) has been identified as best approach with high fPOM recovery and low cross-contamination. As a consequence, a modification of common USD practice by replacing the *fill-up* with the *trickling* procedure is suggested.

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Author contribution

Frederick Büks developed and conducted the experiment, analyzed the data and prepared the manuscript.

Competing interests

The author declares that he has no conflict of interest.

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