# Effect of air-drying pre-treatment on the characterization of forest soil carbon pools

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1. Introduction

Globally, soils contain more than 2500 Pg carbon (C) and therefore store 3-4 times as much C as the land biota (560-654 Pg) and the atmosphere (845 Pg) ([Batjes, 1996](#_ENREF_3); [Lal, 2004](#_ENREF_38); [Prentice et al., 2001](#_ENREF_44); [Tarnocai et al., 2009](#_ENREF_62)). However, the future of the amount of C stored in soils worldwide and the potential of soil to act as net CO2 sink under global warming scenarios is still highly uncertain ([Cox et al., 2000](#_ENREF_14); [Smith and Fang, 2010](#_ENREF_57)). Measuring and verifying changes in soil C stocks and unraveling the factors controlling long-term soil carbon stability are still major challenges that compromise our understanding of the global carbon cycle ([Jandl et al., 2014](#_ENREF_29); [Rumpel et al., 2004](#_ENREF_51); [Saby et al., 2008](#_ENREF_52)).

Archived soils could represent a valuable resource for the spatio-temporal inventory of soil carbon stability. For instance, long-term field experiments and soil collections (e.g. the Rothamsted Archive; http://www.rothamsted.ac.uk/sample-archive) have been the main source of data in the parameterization of soil C models ([Coleman and Jenkinson, 1995](#_ENREF_13)) that ultimately have been able to predict potential C sequestration rates under different land uses, management and climatic conditions ([Peltre et al., 2012](#_ENREF_43); [Smith et al., 1997](#_ENREF_58)). Worldwide, there are many other research centers and organizations holding valuable archives ([Chapman et al., 2013](#_ENREF_9); [De Nobili et al., 2006](#_ENREF_15); [Jandl et al., 2014](#_ENREF_29); [Richter et al., 1999](#_ENREF_47); [Torn et al., 2002](#_ENREF_64)), including not only temporal but also extensive spatial soils collections spanning, in some cases, a continental scale ([Baritz et al., 2010](#_ENREF_2); [Karssies et al., 2011](#_ENREF_35)). However, the use of soil samples from archives can present some complications in relation to the type of pretreatment undertaken and the soil parameters of interest. Drying is one of the most common pretreatments to ensure that samples are stored in a relatively stable state ([De Nobili et al., 2006](#_ENREF_15); [Karssies et al., 2011](#_ENREF_35)). Although drying pretreatment is thought not to significantly change Total Carbon (TC) and Total Organic Carbon (TOC) measurements ([Blake et al., 2000](#_ENREF_6)), it may alter soil properties such as pH, total S, extractable nutrients, microbial biomass and respiration ([De Nobili et al., 2006](#_ENREF_15); [Koopmans and Groenenberg, 2011](#_ENREF_37)), and also the quantity and quality of labile C pools obtained through cold-water extractions ([Jones and Willett, 2006](#_ENREF_30); [Kaiser et al., 2001](#_ENREF_31); [Merckx et al., 2001](#_ENREF_42); [Qualls and Haines, 1991](#_ENREF_45)).

It is well-known that soil C should not only be assessed in terms of total concentration but also with respect to its quality and expected stability ([Lal, 2004](#_ENREF_38); [Rumpel et al., 2004](#_ENREF_51)). Thus, several types of chemical extraction procedures and physical fractionation schemes have been developed with the aim of isolating C fractions differing in their chemical nature ([Balaria et al., 2009](#_ENREF_1); [Ghani et al., 2003](#_ENREF_24)) and degree of physical protection ([Six et al., 2004](#_ENREF_55); [Sohi et al., 2001](#_ENREF_59)), while also exhibiting differences in stability and turnover rates ([Gregorich et al., 2006](#_ENREF_25); [von Lützow et al., 2007](#_ENREF_67)). Cold and hot-water extractions have been mainly used to determine readily decomposable fractions of soil organic matter (monomeric carbohydrates, aliphatic acids, low molecular weight phenols, free amino acids and peptides) and thus are used to define labile soil organic carbon (SOC) pools ([Balaria et al., 2009](#_ENREF_1); [Gregorich et al., 2006](#_ENREF_25); [Landgraf et al., 2006](#_ENREF_39)). In contrast, chemical extractions (e.g. NaOCl, H2O2, Na2S2O8, HCl) have not yet been successful in the exclusive isolation of stable C fractions due to the preferential isolation of significant proportions of young C ([Helfrich et al., 2007](#_ENREF_27)). While chemical extraction approaches are based on inherent chemical recalcitrance, physically based fractionation schemes examine physical stabilization of SOC in aggregates and/or by mineral complexation ([Six et al., 2002](#_ENREF_56); [Sohi et al., 2001](#_ENREF_59); [von Lützow et al., 2007](#_ENREF_67)). Physical and chemical methods, given that they represent different stabilization mechanisms, can be partly complementary. Some of the above methods ([Ghani et al., 2003](#_ENREF_24); [Sohi et al., 2001](#_ENREF_59)) were developed for use on fresh field-moist samples and the effect of a drying pretreatment on soil C quality is not commonly assessed. Therefore, it is uncertain if C quality as estimated by fractionation of dried samples provides a good representation of that in a freshly-sampled equivalent soil sample and this undermines the application of such methods to dried soils, and consequently most archived soils.

Thus, the aim of this study was to evaluate the effect of an air-drying pretreatment on: i) the amount and the composition of two water soluble C fractions (cold- and hot-water extractable C) and ii) the concentration of C in physical fractions isolated by density flotation and particle size fractionation from forest soils under different tree species and with distinct soil properties. We focus on forest soils because of their importance as a store of C ([Baritz et al., 2010](#_ENREF_2); [Prentice et al., 2001](#_ENREF_44)) and their suggested relative sensitivity to drying treatment due to their protection under field conditions from rapid changes in soil moisture content by the moderating influence of the forest canopy and litter layer ([Černohlávková et al., 2009](#_ENREF_8); [Fierer and Schimel, 2002](#_ENREF_22)).

2. Materials and methods

2.1. Study site, soil sampling and pre-treatment

The sites were located within the Alice Holt Forest in Farnham (Surrey, South-East England). Alice Holt Forest has been a Forestry Commission-managed forest since 1924 and covers an area of 769 ha (National Grid reference SU 813427). The climate is characteristic of south-east England, with a mean annual (1994-2006) rainfall of 803 mm and average annual temperature of 10.1 oC ([Benham, 2008](#_ENREF_4)). The experimental area includes a wide range of temperate forest ecosystems, with two main distinctive forest types: second-growth forests of conifers (377 ha) mainly dominated by stands of Corsican Pine (*Pinus nigra* var. maritima) and old-growth woodlands (392 ha) of oak (*Quercus robur* and *Quercus petraea*), beech (*Fagus sylvatica*) and mixed broadleaves with stands of up to 200 years old. Three different types of forest were selected that were representative of major forest soil types and tree species in the UK ([Vanguelova et al., 2013](#_ENREF_65)): a gleysol under oak (*Q. robur*; S1), a cambisol under beech (*F. sylvatica*; S2) and a cambisol under pine (*P. nigra*; S3). Triplicate soil samples from each forest type were collected by horizon (O, A and B) in December 2009 and basic physico-chemical properties are given in **Table 1**. Field moisture contents were 60-70% for organic horizons, 20-30% for A horizons and 19-21% for B horizons. Soil samples were sieved field moist to either 2 mm (mineral horizons; isolating small macroaggregates (0.25–2 mm) and microaggregates (53-250 µm)) or 4 mm (organic horizons) and sub-samples of soils from each forest type and horizon were air dried at room temperature (25 oC) for two weeks (‘dry samples’), while the remaining soils, designated ‘field moist samples’, were stored in the field moist state at 4 oC. These air-dried and field moist samples were subject to cold- and hot-water extraction and physical fractionation as described below.

2.2 Cold-water extractable carbon (CWEC) and hot-water extractable carbon (HWEC)

A sequential cold-water and hot-water extraction using a method modified from ([Ghani et al., 2003](#_ENREF_24)) was carried out. Briefly, soil samples (3-4 g, oven dry equivalent) in 50 mL polypropylene centrifuge tubes were extracted with 30-40 mL (1:10 solid-to-liquid ratio) of ultrapure water (>15 Ω Purelab Maxima) by shaking end-over-end for 3h at 20 oC. The tubes were then centrifuged (20 min, 15,000 rpm) and the supernatant filtered through 0.45 µm cellulose nitrate membrane filters to obtain the cold-water extractable C (CWEC). A further 30-40 mL of ultrapure water (1:10 solid:liquid) was then added to the soil pellet and tubes were vortexed in order to bring all solids into suspension and placed in a water bath at 80 oC for 16 h. The resulting extracts were centrifuged and filtered as described previously for CWEC, in order to obtain the hot-water extractable C (HWEC). Total, inorganic and organic C were measured in both cold-water and hot-water extracts with a Shimadzu TOC-5000 analyser using high temperature and catalytic oxidation.

In order to assess changes in the quality of the water extractions the phenolic content of both CWEC and HWEC extractions was measured by colorimetric spectrometry using the Folin-Ciocalteu method ([Folin and Ciocalteu, 1927](#_ENREF_23)), modified by Kalbitz *et al.* ([2008](#_ENREF_32)). Changes in the phenolic content after drying might indicate that the samples had undergone strong biochemical (polymerization/depolymerization of phenolic molecules; ([Sinsabaugh, 2010](#_ENREF_54))) and physicochemical (altered organomineral sorptive interactions; ([Kaiser et al., 2001](#_ENREF_31))) changes during pre-treatment. Briefly, 100 µL of Folin-Ciocalteu´s reagent (Sigma Aldrich, No F9252), 200 µL of saturated Na2CO3 solution, 300 µL ultrapure water and 1.4 mL aliquots of sample were added to 2 mL Eppendorf tubes and vortexed to mix. Samples were incubated for 20 min at room temperature and the extent of blue-coloured complex development in the presence of phenolic compounds was determined by measurement of absorbance at 725 nm against salicylic acid standard solutions (0-40 mg L-1). The concentration of phenols in CWEC and HWEC extractions was expressed as milligrams of salicylic acid equivalents per litre (mg eq L-1) and as a percentage of the total soluble C for each extraction (60.9% C per molecule of salicylic acid).

2.3. Density and particle-size fractionation

Physical C fractions were obtained by a density and particle-size fractionation ([Sohi et al., 2001](#_ENREF_59)) on the mineral topsoil (A horizon) samples. Organic horizons were not included in this experiment because there is not yet a physical fractionation method designed to be conducted on soil of organic nature. Samples from the A horizon were chosen because, after those from the O horizon, they contained the highest proportion of C and, out of the mineral horizons, this horizon has the highest variability in C due to annual but also long-term changes in C inputs and outputs ([Benham et al., 2012](#_ENREF_5)) (**Table 1**). The method of Sohi et al. combines density flotation on sodium iodide (NaI) to isolate ‘light’ C of low density (<1.80 g cm-3) from ‘heavy’ (organo-mineral associated) C, ultrasonic dispersion to release intra-aggregate light C and particle size fractionation of the heavy fraction. The method yields two light fractions (free and intra-aggregate) and four organo-mineral fractions (coarse sand, fine sand, coarse silt, fine silt and clay). Before the fractionation, both dry and field moist samples were brought to field capacity by placing them on a tension table that was at a suction of ~1 m. This was to start the fractionation with soils of equal matric potential as otherwise soils of different moisture content could have changed the density of the NaI solution by dilution and a fixed density of NaI is critical for the physical fractionation of the light fractions. A modification of the method was also introduced in order to increase the total amount of soil under fractionation (from 15 to 30 g). A sensitivity study was conducted in advance to determine the ultrasonic energy output, speed and time of centrifugation that was required to keep the same final proportional distribution of carbon (mass basis) between the fractions as that produced by the standard method based on 15 g soil. The final modified protocol is as follows. Rewetted soils (30 g; n = 3) were mixed with 180 mL of NaI (1.80 g cm-3) into 250 mL polycarbonate centrifuge bottles, shaken by hand (30 sec) and centrifuged (30 min; 8000 g). For each sample, the floating soil material was collected with a pipette attached to a vacuum system and filtered through 2 µm pore size glass fiber filter (Whatman GF/A). The filtered sample (Free Light Fraction= FLF) was washed with ultrapure water and dried at 50 oC. The soil pellet was resuspended in the NaI filtrate collected from the first extraction and the aggregates were dispersed by applying 1500 J of ultrasonic energy per gram of soil (the ultrasonic probe was calibrated by temperature changes in cold water). The centrifugation and filtration of the next fraction (Intra-aggregate Light Fraction= IALF) was then conducted as explained before for the FLF. The two density separation steps were followed by a particle-size fractionation of the soil residue. Four different organo-mineral fractions were separated by flushing water through a wet sieving machine (Endecott Test Sieve Shaker): Coarse Sand Fraction (CSF= 2000-212 µm diameter), Fine Sand Fraction (FS= 212-53 µm), Coarse Silt Fraction (CST=53-25 µm), and Fine Silt and Clay Fraction (SC < 25 µm). Each organo-mineral fraction, apart from the SC fraction, was collected by washing the corresponding sieve with ultra-pure water. The SC fraction, which was the suspension’s residual, was collected after the wet sieving step and was isolated by flocculation (2.5 mL of 0.1 M CaCl2 x 250 mL of washing) and centrifugation (2500 g for 30 min). All organo-mineral fractions were oven dried (50 oC) and the TOC measured by dry combustion (Carlo Erba Flash 1112). C recovery (sum of the mass of C in each fraction expressed as a percentage of the mass in the unfractionated soil) following this procedure ranged from 90 to 103%. An in house reference material for the TOC analysis traceable to certified reference soil GBW07412 (State Bureau of Technical Supervision, The People's Republic of China) was also included (recoveries: 96-105%).

2.4. Statistical analysis:

Data were tested for normality and log transformed when required. Two-way Anova was used to test the effect of air-drying pretreatment (dry or fresh), forest type (S1, S2 and S3) and their interactions, as appropriate. The simple least significant difference (LSD) at three levels of probability (0.05, 0.01 and 0.001) was used for comparisons of individual means between fresh field moist and dry samples in cases where the ANOVA revealed a significant effect of pretreatment as a main effect and/or in interaction with other factors. These statistical analyses were conducted using Genstat 11th Edition (VSN International Ltd, Hemel Hempstead, UK).

3. Results & Discussion

3.1 Effect of air-drying on the quantity and quality of cold- and hot-water extractable C

The concentration of carbon extracted from the air-dried and field moist study soils using sequential cold- and hot-water extractions is shown in **Figure 1**. The concentrations of organic carbon extracted by hot water (493-5047 mg kg-1) were, on average, 4.5 times higher than those extracted by cold water (123-883 mg kg-1). These results reflect the increased efficiency of the 4-fold higher temperature and longer incubation time (16 h *cf.* 3 h) of the hot- water extraction in bringing organic carbon compounds additional to the CWEC into solution (**Figure 1:a,b**). Likewise, Landgraf ([2006](#_ENREF_39)) reported a 3-5 times increase in the amounts of HWEC when compared to CWEC solutions extracted from O horizons of a 170-year-old beech German forest. In our study, whilst the CWEC represented only a small component (0.78-1.6%) of the total SOC, the HWEC represented up to 5.3%, with mineral horizons having the largest proportions of both cold- and hot-water extractable C (**Figure 1:c,d**). The proportions of total water extractable C were within the range of values reported previously for temperate forest soils (0.50-2.50% for CWEC and 0.93-11.4% for HWEC) ([Balaria et al., 2009](#_ENREF_1); [Boyer and Groffman, 1996](#_ENREF_7); [Chen et al., 2004](#_ENREF_10); [Chodak et al., 2003](#_ENREF_11); [Landgraf et al., 2006](#_ENREF_39)) with some of these studies ([Boyer and Groffman, 1996](#_ENREF_7); [Chodak et al., 2003](#_ENREF_11)) reporting an increase in solubility in mineral horizons in comparison to organic top layers which is also in agreement with the current findings.

Air-drying pretreatment had an overall highly significant (p < 0.01) effect on the concentration of cold-water extractable C in organic and mineral horizons across the three different forest sites (**Table 2**), with the concentration of C extractable from dry mineral horizon samples up to two-fold greater than that extractable from field moist soils (**Figure 1a**). When forest site was included as a source of variation for CWEC concentration, this factor was also found to be statistically significant (**Table 2**). The effect of an air-drying pretreatment on HWEC was significant as a main factor for Horizon B (p < 0.01) and in interaction with forest site for Horizon O and A (**Table 2**). Unlike the CWEC (**Figure 1a**), pair-wise comparisons between HWEC for air-dried and field moist samples within forest type and horizon were not always significant (**Figure 1b**).

An increase in CWEC has been extensively reported when soils are exposed to droughts in the field ([Kalbitz et al., 2000](#_ENREF_33)) but also after soil drying under controlled laboratory conditions ([Christ and David, 1996](#_ENREF_12); [Jones and Willett, 2006](#_ENREF_30); [Klitzke and Lang, 2007](#_ENREF_36); [Koopmans and Groenenberg, 2011](#_ENREF_37); [Merckx et al., 2001](#_ENREF_42)). For example, Jones et al., ([2006](#_ENREF_30)) have found a three-fold increase in CWEC when comparing field moist and air dried mineral soil samples (30 oC for 24 h) under different land uses (forests and grasslands). Koopmans & Groenenberg ([2011](#_ENREF_37)) reported a 1.7-fold increase, when comparing the amount of C solubilized by an aqueous solution of Ca(NO3)2 (2 mM, 24 h;20 oC) from field moist samples of a sandy forest soil (Tilburg forest park, The Netherlands) to the corresponding oven-dried samples (40 oC). Many authors, based on the analysis of DOC composition, have attributed this increased production of dissolved organic C to be a result of cell lysis under drought stress and production of cell exudates due to osmotic shock after rewetting ([Christ and David, 1996](#_ENREF_12); [Halverson et al., 2000](#_ENREF_26); [Zsolnay, 2003](#_ENREF_68)). Thus, it is likely that following air-drying, the CWEC contained biomass C that was intact and non-extractable in corresponding fresh soils, explaining the difference in CWEC concentration between fresh and air-dry treatments.

Unlike cold-water extractions, hot-water extractions make the microbial biomass component soluble, since temperatures around 70 oC are supposed to be sufficient to lyse most vegetative microbial cells ([Sparling et al., 1998](#_ENREF_61)). In fact, the microbial biomass pool is considered to be a quantitatively important component of the HWEC, accounting for about 40% of its C composition ([Balaria et al., 2009](#_ENREF_1); [Ghani et al., 2003](#_ENREF_24)). Under the assumptions that (i) hot-water extraction solubilises biomass C; and, (ii) a proportion of the biomass initially present in fresh soil was rendered cold-water extractable following drying, it might be expected that, when used sequentially, hot-water extraction would extract less C from air-dried soils than respective fresh soils due to the greater extraction, during the cold water step, of C that would be hot-water extractable. Whilst lower HWEC concentrations in air-dried samples were found for some horizons (e.g. Oak (S1) horizon B) when compared to field moist samples, this was not a consistent finding across all pair-wise comparisons (**Figure 1b**), possibly reflecting the small concentration of (biomass) C rendered cold-water extractable by air-drying relative to the size of the total HWEC fraction. Examining the total amount of water soluble carbon extracted by both cold- and then hot-extraction (**Figure 2**) revealed that the effect of an air-drying pretreatment was significant as a main factor for Horizon A (p < 0.05) and in interaction with forest site for Horizon O (**Table 2**). However, the majority of the post-hoc pair-wise comparisons of means revealed no effect of pretreatment which is consistent with our interpretation (above) that the (biomass) C that is cold-water extractable from air-dried soil but not from the equivalent fresh sample is eventually sequentially extracted from fresh soils as HWEC.

In addition to effects on the quantity of water extractable C, the air-drying pretreatment also had an impact on the quality of the CWEC with respect to the concentration and relative proportions of phenolic compounds (**Figure 3, Table 3**). On average, the concentration of phenolic compounds extracted by cold water increased 1.7-fold after air-drying the soils, with increases being more pronounced for A horizons (up to 4-fold) than O and B horizons (1.1-1.7 and 1.2-3.1-fold, respectively). The effect of air-drying on the phenolic composition of HWEC was only significant for O horizon samples when in interaction with forest site (**Figure 3, Table 3**). The release of cold water-extractable phenolic compounds on air drying could be due to microbial cell disruption and release of intracellular solutes as previously mentioned. Specifically, drought stress and soil rewetting can induce the release of microbial monomers ([Halverson et al., 2000](#_ENREF_26)), some of which (e.g. tyrosine, tryptophan, guanine) are reactive to the Folin-Ciocalteu reagent used in the colorimetric assay for phenols ([Everette et al., 2010](#_ENREF_20)). On the other hand, a process of air-drying could increase the release of humified material, and therefore phenolic compounds, into the soil solution during the drying process ([Zsolnay et al., 1999](#_ENREF_69)): 1) by increasing the breakdown of H-bonds within organic polymeric molecules that were formed in the presence of water and 2) through an enhancement in the rate of oxidation of the organic matter due to increases in temperature (25 oC *cf* 4 oC) and greater exposure to oxygen after water evaporation ([Raveh and Avnimelech, 1978](#_ENREF_46))..

3.2 Effect of air-drying on physical SOC fractions

As shown in **Figure 4** and **Table 4**, the concentration of C in the free-light fraction (FLF) was generally higher in air-dry samples (overall average 1.65 ±1.26 g C kg-1 soil) than in field-moist samples (1.13±0.16 g C kg-1 soil) (**Figure 4a**), while the opposite results were found for the intra-aggregate light fraction (IALF; **Figure 4b**), with field-moist samples showing a greater concentration of C associated to this fraction (field-moist: 3.65±0.24 g C kg-1; air-dry: 2.8±0.15 g C kg-1). The air-drying pretreatment had a significant effect for the coarse sand (CSD) and, in interaction with forest site, the fine sand (FS) fraction (**Figures 4c and 4d**) while no significant effects were found for the coarse silt (CST), and the fine silt+clay fractions (SC) (**Figures 4e and 4f**; **Table 4**).

The process of drying is known to increase the hydrophobicity of soil samples ([Dekker et al., 2001](#_ENREF_17); [Rodríguez-Alleres and Benito, 2012](#_ENREF_48)), consequently increasing their resistance to be rewetted due to an enhanced water repellency ([Doerr and Thomas, 2000](#_ENREF_19)). The degree of water repellency can be significantly more pronounced in forest soils than other types of land uses ([McGhie and Posner, 1981](#_ENREF_41); [Rodríguez-Alleres et al., 2007](#_ENREF_49)), in part due to the greater abundance of plant litter remains, which are major sources of hydrophobic substances in soils ([Doerr and Thomas, 2000](#_ENREF_19); [Rodríguez-Alleres et al., 2007](#_ENREF_49)). Thus, a drying pretreatment may be expected to impact the hydrophobicity of undecomposed plant materials, or by analogy free-light C fractions, to a higher extent than other soil components ([Six et al., 2004](#_ENREF_55)). As a result, the rates of rewetting will be reduced for this particular fraction, which ultimately could also decrease its density in the fractionation procedure due to a greater proportion of air encapsulation into the plant micropores. Thus, not only an increase in the hydrophobicity of the FLF but also a subsequent density reduction could lead to lower sedimentation rates after and during the first step of centrifugation, explaining thus the greater C recoveries found for this fraction after air-drying. On the other hand, it is also possible that air drying pretreatment released some of the IALF-associated C into the FLF due to a decrease in aggregate stability after drying ([Denef et al., 2001](#_ENREF_18); [Rovira and Greacen, 1957](#_ENREF_50); [Soulides and Allison, 1961](#_ENREF_60)) and destabilization prior to or during the FLF isolation step. Our data (**Figure 4a and b**), which shows an overall decrease in IALF-associated C for air dried soils that corresponds to the increase in FLF-C, is supportive of such a release of light fraction from within aggregates and its subsequent isolation as FLF.

Regarding the impact of an air-drying pretreatment on the amount of organo-mineral associated C, although significant differences between air-dry and field-moist samples were also found for the amount of C bound to sand-size mineral fractions (**Table 4**), the pair-wise comparisons were in many cases not significant and there was no clear trend regarding the effect of the drying pretreatment (**Figure 4**). Among all physical C fractions, SC was the soil fraction with the highest C contents, with an overall mean of 23.46 g C kg-1 soil and representing 45-75% of the total organic C in the A horizon. Similar proportions of SC-C were also found for the A horizon of two long-term experimental woodlands at Rothamsted Research in southeast England, reaching in both cases about 50% of the TOC ([Tonon et al., 2010](#_ENREF_63)). However, even higher percentages of C in the SC fraction (63-91%) have been reported for mineral horizons in luvisols and cambisols under forest land uses in east Germany ([Schöning and Kögel-Knabner, 2006](#_ENREF_53)). As illustrated by our data and by the findings reported in studies discussed above, C associated to silt and clay fractions is in general quantitatively more important than sand fractions, since the C contents of heavy fractions tend to increase with decreasing particle size ([Hinds and Lowe, 1980](#_ENREF_28); [Kandeler et al., 1999](#_ENREF_34)). During late decomposition, sorption to mineral surfaces (through i.e. ligand exchange, polyvalent cation bridges, hydrophobic and weak interaction) reduces utilization by microorganisms and therefore decomposition rates ([von Luetzow et al., 2006](#_ENREF_66)). Small particles such as fine silt and clay-sized particles provide numerous reactive sites and larger surface areas where organic matter sorption takes place ([von Luetzow et al., 2006](#_ENREF_66)). Among all physically-derived soil fractions, the fine silt and clay fractions are considered the most stable and resilient due to their longer soil carbon turnover rates ([Rumpel et al., 2004](#_ENREF_51); [Schöning and Kögel-Knabner, 2006](#_ENREF_53)). Our analysis revealed no significant effect of pretreatment on the concentration of SC- associated C when analysed both as a single factor or partitioned by forest sites (**Table 4**), and is thus supportive of the resilience of this fraction to soil perturbation.

4. Conclusions

In order to evaluate air-drying as a soil treatment prior to characterization of soil organic carbon quality, we applied fractionation methods to examine the distribution of soil organic carbon between physically- and chemically-defined fractions in air-dried and corresponding field-moist forest soils. The experimental data suggest that HWEC was less impacted by an air drying pretreatment than the CWEC. This is probably because CWEC is more sensitive to an air-drying mediated release of microbial biomass C lysate, whereas HWEC, which already accounts for biomass-derived C, remains practically unaltered. Thus, HWEC is recommended as an extraction that is more robust to an air drying pre-treatment and consequently can be applied to air-dried soils to produce data on labile soil C that is more translatable to soil in the field-moist state.

In comparison to fresh samples, physical fractionation of air-dried samples over-estimated the FLF and under-estimated the IALF. However, the method was particularly robust when applied to the silt and clay fraction, supporting its application to dried soil samples (including potentially those in soil archives) to provide data on long-term C sequestration potential that is again more translatable to soil in the field-sampled state.

Our conclusions are reached on the basis of experiments on gleysol and cambisol forest soils that are representative of major soil types in the UK, Europe ([De Vos et al., 2015](#_ENREF_16)) and globally ([FAO, 2015](#_ENREF_21)). The assessment of soil carbon stocks and quality is also of considerable interest and importance for soils under non-forestry land use (e.g. arable and pasture lands; ([Lugato et al., 2014](#_ENREF_40))) and therefore further soil and land use types should be tested to evaluate the wider application of our findings.

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TABLES

**Table 1**Physicochemical properties of the organic and mineral horizons of the three forest sites (S1- gleysol under *Quercus robur*, S2- cambisol under *Fagus sylvatica*, S3- cambisol under *Pinus nigra*). Data are the mean and standard error in parenthesis (n =3).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Site | H. † | SOM ‡  (%) | TC (%) § | TN (%) § | C/N | pH ¶ | Particle Size Analysis (%) # | | |
| Sand | Silt | Clay |
|  |  |  |  |  |  |  |  |  |  |
| S1 | O | 59.7 (0.5) | 30.7 (0.2) | 1.6 (0.1) | 19.3 | 3.9 (0.1) |  |  |  |
| A | 10.8 (0.2) | 4.9 (0.1) | 0.4 (0.1) | 13.5 | 3.2 (0.1) | 20.8 | 69.2 | 9.9 |
| B | 5.5 (0.1) | 2.1 (0.1) | 0.2 (0.1) | 13.4 | 3.8 (0.1) | 14.2 | 72.2 | 13.6 |
| S2 | O | 41.8 (1.1) | 22.9 (0.8) | 1.2 (0.1) | 18.9 | 3.4 (0.1) |  |  |  |
|  | A | 6.6 (0.1) | 3.4 (0.8) | 0.2 (0.1) | 14.5 | 3.0 (0.1) | 38.1 | 56.0 | 6.0 |
|  | B | 5.2 (0.1) | 2.4 (0.1) | 0.2 (0.1) | 15.1 | 3.6 (0.1) | 43.7 | 50.0 | 6.3 |
| S3 | O | 81.1 (0.2) | 44.8 (0.4) | 1.9 (0.1) | 23.4 | 2.9 (0.1) |  |  |  |
|  | A | 8.4 (0.1) | 4.1 (0.1) | 0.3 (0.1) | 15.5 | 3.0 (0.1) | 42.3 | 52.3 | 5.4 |
|  | B | 4.9 (0.7) | 2.1 (0.1) | 0.2 (0.1) | 12.6 | 3.3 (0.1) | 36.0 | 55.8 | 8.2 |
| †: Horizon. ‡: Soil Organic Matter (SOM) was determined gravimetrically by loss on ignition in a muffle furnace at 500 oC for 12h. §: Total Carbon (TC) and Total Nitrogen (TN) contents were analysed by combustion with a LECO SC-444 autoanalyser and Europe Roboprep-VG 662 , respectively. ¶: pH was determined in aqueous extracts (1:10 for organic and 1:5 for mineral horizons). #: Particle size analysis of the mineral soil samples was carried out by using a Coulter LS Particle Size Analyzer. | | | | | | | | | |

**Table 2** Analysis of variance (F-value) for the amount (mg kg-1) and proportion (% of TOC) of cold water-extractable C (CWEC), hot water-extractable C (HWEC) and CWEC+HWEC for field-moist and air-dried samples. d.f. = degrees of freedom; ns = non significant (p-value given between brackets); \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. † Residual variance within factors (mean square).

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |  |
|  | **d.f.** | **CWEC** | |  | **HWEC** | |  | **CWEC+HWEC** |
|  |  | **(mg kg-1)** | **% of TOC** |  | **(mg kg-1)** | **% of TOC** |  | **(mg kg-1)** |
|  |  |  |  |  |  |  |  |  |
| ***Horizon O*** |  |  |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 13.06\*\* | 4.32ns(0.060) |  | 0.37ns(0.553) | 1.18ns(0.299) |  | 0.83ns(0.381) |
| Forest Site (F) | 2 | 660.25\*\*\* | 818.37\*\*\* |  | 18.69\*\*\* | 14.74\*\*\* |  | 23.32\*\*\* |
| P x F | 2 | 163.28\*\*\* | 99.85\*\*\* |  | 10.56\*\* | 5.02\* |  | 11.84\*\* |
|  |  |  |  |  |  |  |  |  |
| *Residual Variance*† | 12 | 221.80 | 0.01 |  | 0.11 | 0.02 |  | 0.01 |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
| ***Horizon A*** |  |  |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 611.24\*\*\* | 893.78\*\*\* |  | 4.68ns(0.051) | 6.88\* |  | 7.43\* |
| Forest Site (F) | 2 | 11.72\*\* | 352.23\*\*\* |  | 39.99\*\*\* | 210.11\*\*\* |  | 31.35\*\*\* |
| P x F | 2 | 15.27\*\*\* | 15.57\*\*\* |  | 4.90\* | 5.19\* |  | 4.21\* |
|  |  |  |  |  |  |  |  |  |
| *Residual Variance* † | 12 | 554.70 | 0.01 |  | 0.99 | 0.05 |  | 5.90 |
|  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |
| ***Horizon B*** |  |  |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 1081.09\*\*\* | 1131.45\*\*\* |  | 16.48\*\* | 15.38\*\* |  | 0.39ns(0.545) |
| Forest Site (F) | 2 | 256.52\*\*\* | 209.70\*\*\* |  | 39.90\*\*\* | 39.22\*\*\* |  | 67.08\*\*\* |
| P x F | 2 | 24.62\*\*\* | 29.66\*\*\* |  | 1.77ns(0.212) | 1.35ns(0.297) |  | 3.71ns(0.056) |
|  |  |  |  |  |  |  |  |  |
| *Residual Variance* † | 12 | 70.12 | 0.01 |  | 0.34 | 0.04 |  | 0.35 |
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**Table 3** Analysis of variance (F-value) for the concentration (mg eq L-1) and proportions (as % of water extractable C) of phenolic compounds of CWEC and HWEC for field-moist and air-dried samples. ns = non significant (p-value given between brackets) ; \* = p < 0.05; \*\* = p < 0.01;\*\*\* = p < 0.001. † Residual variance within factors (mean square).

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| --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |
|  |  | **Phenolic Content CWEC** | |  | **Phenolic Content HWEC** | |
|  | **d.f.** | **mg eq L-1** | **% of WEC** |  | **mg eq L-1** | **% of WEC** |
|  |  |  |  |  |  |  |
| ***Horizon O*** |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 458.64\*\*\* | 222.71\*\*\* |  | 0.01ns(0.995) | 0.27ns(0.610) |
| Forest Site (F) | 2 | 1046.09\*\*\* | 72.70\*\*\* |  | 115.03\*\*\* | 39.31\*\*\* |
| P x F | 2 | 81.30\*\*\* | 25.34\*\*\* |  | 35.97\*\*\* | 4.40\* |
|  |  |  |  |  |  |  |
| *Residual Variance* † | 12 | 0.01 | 1.15 |  | 273.30 | 4.87 |
|  |  |  |  |  |  |  |
| ***Horizon A*** |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 950.87\*\*\* | 218.97\*\*\* |  | 0.20ns(0.665) | 10.05ns(0.008) |
| Forest Site (F) | 2 | 70.13\*\*\* | 43.36\*\*\* |  | 0.860.447 | 57.39\*\*\* |
| P x F | 2 | 13.73\*\*\* | 4.34\*\*\* |  | 3.55ns(0.061) | 3.38ns(0.068) |
|  |  |  |  |  |  |  |
| *Residual Variance* † | 12 | 0.71 | 1.15 |  | 14.90 | 0.52 |
|  |  |  |  |  |  |  |
| ***Horizon B*** |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 267.27\*\*\* | 31.90\*\*\* |  | 1.00ns(0.337) | 3.47ns(0.087) |
| Forest Site (F) | 2 | 234.90\*\*\* | 106.22\*\*\* |  | 122.70\*\*\* | 121.68\*\*\* |
| P x F | 2 | 81.63\*\*\* | 21.67\*\*\* |  | 0.38ns(0.693) | 0.62ns(0.553) |
|  |  |  |  |  |  |  |
| *Residual Variance* † | 12 | 0.37 | 1.97 |  | 3.09 | 0.90 |
|  |  |  |  |  |  |  |
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**Table 4** Analysis of variance (F-value) for the six physical fractions (g C kg-1) measured on the A mineral horizon at the three forest sites (S1, S2 and S3) for field-moist and air-dried samples. ns = non significant (p-value given between brackets) ; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. † Residual variance within factors (mean square).

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| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  |
|  | **d.f.** | **FLF** | **IALF** | **CSD** | **FS** | **CST** | **SC** |
|  |  |  |  |  |  |  |  |
| ***Two-way Anova*** |  |  |  |  |  |  |  |
| Air-drying Pretreatment (P) | 1 | 19.65\*\*\* | 79.13\*\*\* | 15.39\*\* | 0.03ns(0.866) | 3.12ns(0.102) | 0.07ns (0.789) |
| Forest Sites (F) | 2 | 166.55\*\*\* | 56.28\*\*\* | 1427.51\*\*\* | 129.24\*\*\* | 1.06ns(0.376) | 44.95\*\*\* |
| P x F | 2 | 0.99ns (0.401) | 9.69\*\* | 12.73\*\* | 7.23\*\* | 1.86ns(0.197) | 0.93ns (0.423) |
|  |  |  |  |  |  |  |  |
| *Residual Variance*† | 12 | 0.06 | 0.0006 | 0.005 | 0.122 | 0.6818 | 3.59 |
|  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |

FIGURES



**Figure 1**The effect of an air-drying pretreatment on water extractable soil C using a cold water extraction-CWEC (a,c) and, sequentially, a hot water extraction-HWEC (b,d) from the O, A and B horizons of three forest soils (S1, S2 and S3). The data are expressed as total concentration of dissolved organic carbon (mg kg-1; a,b) and as % of the TOC (c,d). Different letters indicate statistically significant differences between field moist and dry samples within site and horizon (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. Error bars show the standard errors of the mean (n=3).



**Figure 2** The effect of an air-drying pre-treatment (F: field moist and D: air-dried) on total water extractable organic C (CWEC+ HWEC) concentrations (mg kg-1) for the O, A and B horizons of three forest soils (S1: gleysol under oak, S2: cambisol under beech and S3: cambisol under pine). ns = non significant; \* = p<0.05; \*\* = p < 0.01; \*\*\* = p < 0.001 for post-hoc pair-wise comparisons of field moist versus dry samples within site and horizon. There was no significant effect of pretreatment as a main effect or in interaction with site for B horizon samples (Table 2) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).

Figure 3.tif

**Figure 3**  The effect of an air-drying pretreatment on total phenolics [expressed as mg eq L-1 (a,b) and % of TOC (c,d)] in cold water (a,c) and hot water (b,d) soil extracts from the O, A and B horizons of three forest soils (S1, S2 and S3). Different letters indicate statistically significant differences between field moist and dry samples within site and horizon (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. There was no significant effect of pretreatment as a main effect or in interaction with site for A and B horizon HWEC samples (Table 3) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).



**Figure 4**The effect of an air-drying pretreatment on the concentration of C in six physical C fractions (g C kg-1 soil): a) Free Light Fraction (FLF), b) Intra-aggregate Light Fraction (IALF), c) Coarse Sand (CSD), d) Fine Sand (FS), e) Coarse silt (CST) and f) Fine Silt and Clay Fraction (SC). Different letters indicate statistically significant differences between field moist and dry samples for each site (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. There was no significant effect of pretreatment as a main effect or in interaction with site for CST and SC fractions (Table 4) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).

**FIGURE CAPTIONS**

**Figure 1**The effect of an air-drying pretreatment on water extractable soil C using a cold water extraction-CWEC (a,c) and, sequentially, a hot water extraction-HWEC (b,d) from the O, A and B horizons of three forest soils (S1, S2 and S3). The data are expressed as total concentration of dissolved organic carbon (mg kg-1; a+b) and as % of the TOC (c,d). Different letters indicate statistically significant differences between field moist and dry samples within site and horizon (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. Error bars show the standard errors of the mean (n=3).

**Figure 2** The effect of an air-drying pre-treatment (F: field moist and D: air-dried) on total water extractable organic C (CWEC+ HWEC) concentrations (mg kg-1) for the O, A and B horizons of three forest soils (S1: gleysol under oak, S2: cambisol under beech and S3: cambisol under pine). ns = non significant; \* = p<0.05; \*\* = p < 0.01; \*\*\* = p < 0.001 for pair-wise comparisons of field moist versus dry samples within site and horizon. There was no significant effect of pretreatment as a main effect or in interaction with site for B horizon samples (Table 2) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).

**Figure 3**  The effect of an air-drying pretreatment on total phenolics [expressed as mg eq L-1 (a,b) and % of TOC (c,d)] in cold water (a,c) and hot water (b,d) soil extracts from the O, A and B horizons of three forest soils (S1, S2 and S3). Different letters indicate statistically significant differences between field moist and dry samples within site and horizon (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. There was no significant effect of pretreatment as a main effect or in interaction with site for A and B horizon HWEC samples (Table 3) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).

**Figure 4**The effect of an air-drying pretreatment on the concentration of C in six physical C fractions (g C kg-1 soil): a) Free Light Fraction (FLF), b) Intra-aggregate Light Fraction (IALF), c) Coarse Sand (CSD), d) Fine Sand (FS), e) Coarse silt (CST) and f) Fine Silt and Clay Fraction (SC). Different letters indicate statistically significant differences between field moist and dry samples for each site (Least Significant Difference Test). ns = non significant; \* = p < 0.05; \*\* = p < 0.01; \*\*\* = p < 0.001. There was no significant effect of pretreatment as a main effect or in interaction with site for CST and SC fractions (Table 4) and therefore no post-hoc analysis was carried out. Error bars show the standard errors of the mean (n=3).