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DISTRIBUTION OF CARBON IN SIZE-FRACTIONS OF A PASTURE SOIL 26 MONTHS AFTER ADDING BIOCHAR

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Abstract

Fractionating soils according to size and/or density of particles improves our understanding of the importance of interactions between organic and inorganic soil components on the turnover of soil organic carbon (SOC). Conventional soil physical fractionation methodologies misrepresent the contribution of pyrogenic C (e.g., biochar-derived C) to the total SOC because of the relative long turnover time of this fraction, regardless the physical SOC physical fraction in which this is found. In this study, a combination of particle size fractionation and wet sieving, as well as chemical analysis (dichromate oxidation) was tested to isolate meaningful SOC fractions in a set of 34 soils with C content ranging from 19.1–43.0 g SOC/kg soil. Topsoil and subsoil samples were obtained after 26 months of simulating cultivation at pasture renewal including pine biochar (10 t/ha) as amendment (below 10 cm depth) and growth of contrasted plant species (ryegrass vs a mixture of red clover and cocksfoot) in a lysimeter experiment using a silt loam soil (Tokomaru soil, a Pallic soil with limited drainage at depth). Across all the soils considered, the allocation of SOC in size-fractions (*i.e.*, 2000-200, 250-53 and <53 μm) was obtained by conventional wet sieving. Additionally, the total content of resistant forms of SOC (*i.e.* both alkyl C forms and pyrogenic C from biochar) was calculated as the sum of the dichromate-resistant C obtained in the different size-fractions. This sum of all dichromate-resistant C pools can be used as a proxy to estimate contribution of pyrogenic C to the total SOC in the soils studied. The different C fractions isolated by the appropriate combination of methodologies (particle size fractionation, wet chemistry) is proposed as an alternative to obtain the particulate, humus and resistant organic carbon fractions (POC, HOC and ROC, respectively) used in models (e.g. RothC). The developed methodology will help to improve the prediction of SOC dynamics and any impact of climate change on SOC stocks when these contain pyrogenic C.

Introduction

Soil organic carbon (SOC) represents a significant reservoir of carbon within the global carbon cycle, comprising up to 1417 Pg (1 Pg = 10^9 tonne) down to 1 m (Batjes, 2014). SOC exists as an heterogeneous mixture of a wide range of organic materials in close association with minerals and with other organic molecules including microbially-processed C (Cotrufo *et al.*, 2015; Liang *et al.*, 2017). Farmers, scientists and policy makers (Minasny *et al.*, 2017; Soussana *et al.*, 2017) are interested in the potential to build SOC content in soils to offset increasing atmospheric carbon dioxide (CO_2) concentrations (Paustian *et al.*, 2016; Smith, 2016). An accurate assessment on the impact of climate change in SOC stocks needs to consider the interactions between all soil components (both organic and inorganic). This heterogeneous nature of SOC-mineral interactions has been studied since long by using a variety of chemical and/or physical fractionation procedures (Christensen, 2001; Poeplau *et al.*, 2013). The final aim of these techniques is the isolation of relatively "homogenous" fractions of SOC (or pools; von Lützow *et al.*, 2007) that may show contrasted functional properties, such as stability and turnover times.

Physical fractionation according to size and/or density of soil particles emphasizes the importance of interactions between organic and inorganic soil components on the turnover of SOC, which is a very dynamic property (Christensen, 2001). Conventional soil physical fractionation methodologies misrepresent the contribution of pyrogenic C (*e.g.*, biochar-derived C) to the total SOC because of the relative long turnover time of this C type (Lehmann *et al.*, 2015), regardless the physical SOC fraction in which pyrogenic C is found (Nocentini *et al.*, 2010).

A group of methodologies used to fractionate soils rely on the partitioning of C into the soil aggregate structure (Six and Paustian, 2014). By a combination of wet sieving, density separation and dispersion, the isolation of aggregate fractions allows to interpret the dynamics of the organic matter in the soil (Six *et al.*, 2002). The method then facilitates the separation of several fractions as: (i) the coarse free particulate organic matter (coarse fPOM), the fine fPOM, physically protected intra-aggregate-POM (iPOM, within microaggregates), (ii) the (silt+clay)-sized organic C outside microaggregates, and (iii) (silt+clay)-sized organic C inside microaggregates. However, the methodology does not attempt to distinguish the presence of pyrogenic C, which can be a drawback when charred material is present (Herath *et al.*, 2014).

A second broad set of methodologies rely on wet sieving for physically fractionate the soil (Baldock *et al.*, 2013b; Curtin *et al.*, 2016). In the end, these methodologies separate the soil into major fractions of contrasted particle-size (*e.g.*, 2000-50, < 50-µm). Baldock *et al.* (2013b), using ^{13}C NMR spectroscopy, quantified the contribution of pyrogenic C to the fractions separated by size in order to obtain three different organic C pools: particulate organic C (POC), humic organic C (HOC), and resistant organic C (ROC). These pools were successfully included in the RothC model (Skjemstad *et al.*, 2004) and novel techniques as visible-near infrared (Vis-NIR) spectroscopy are able to predict fairly accurately the different fractions (Viscarra Rossel *et al.*, 2017). Research on Australian soils (Skjemstad *et al.*, 1996; Skjemstad *et al.*, 1999), as well as worldwide (Reisser *et al.*, 2016), has repeatedly shown the important contribution of pyrogenic C to the total of C in soil hence justifying the inclusion of this C fraction in the ROC pool.

In this study we propose a third methodology, which is a modification of the method from Curtin *et al.* (2016), that incorporates the determination of pyrogenic C by means of dichromate oxidation (Calvelo Pereira *et al.*, 2011; Herath *et al.*, 2014). We used soils sampled 26 months after simulating cultivation at pasture renewal including pine biochar (10

t/ha) as amendment (below 10 cm depth) and growth of contrasted plant species (ryegrass and a mixture of red clover + cocksfoot) in a lysimeter experiment using a silt loam soil (Calvelo Pereira *et al.*, 2016) to test the methodology. We propose that simple wet chemistry methods as dichromate oxidation may be of convenience to identify the contribution of pyrogenic C to the SOC fractions while avoiding issues associated with redistribution of carbon during dispersion and successive manipulation of soils.

Material and Methods

Establishment of the lysimeter trial, experimental design and monitoring

Soils used in this study were obtained from the 2-year lysimeter study of Calvelo Pereira *et al.* (2016). Briefly, a Tokomaru silt loam soil (a Typic Fragiaqualf) developed from wind-blown loess of Greywacke origin was used. A C-rich, low-ash biochar (759 g C/kg biochar, 83% as fixed C; atomic H/C_{org}: 0.62) was produced from pine (*Pinus radiata* D. Don) sawdust and added to test if biochar would overcome the physical constraints of the Tokomaru soil by improving the drainage at the ploughed layer (Calvelo Pereira *et al.*, 2016).

Seventeen PVC pipe lysimeters (PVC columns 40 cm depth and 20 cm in diameter) were set up at Palmerston North, New Zealand, in December 2010 using the Tokomaru soil. The lysimeters were part of a larger experiment simulating biochar incorporation into depth when ploughing the soil for seed bed preparation at cultivation. Soil cores at 0–10, 10–20, and 20–40 cm depth were taken from a pasture site nearby Palmerston North. The 0–10 cm and 10–20 cm depth soil layers were sliced and removed for "cultivation", whereas the 20–40 cm layer was taken intact using the corresponding PVC column. The 0-10 cm layer was hand-mixed with the PI-350 biochar at an application rate of 10 Mg/ha and also with NPK fertiliser. This layer was then added to the soil column in the PVC container on top of the 20–40 cm layer, at a depth of 10–20 cm, thus inverting the order of layering to simulate mouldboard ploughing at pasture establishment (Calvelo Pereira *et al.*, 2016). The original 10–20 cm depth soil was also hand-mixed to simulate the effect of cultivation and added on top of the soil column, at a depth of 0–10 cm, without amendments. 8 columns included PI-350 as amendment (biochar-amended soil treatment) and the other 8 columns did not (nil or non-biochar soil treatment). Once the columns were prepared, the lysimeters were attached to 1.3 m PVC drainage collection flux meters. The trial included an additional column for a destructive sampling at time 0; preparation of this column followed exactly the same "cultivation" as described previously and sampled thereafter for physical and chemical characterisation.

Either perennial ryegrass (*Lolium perenne* L.), or a mixture of red clover (*Trifolium pratense* L.) + cocksfoot (*Dactylis glomerata* L.) were selected as suitable pastures to be planted in the Tokomaru soil. Seeds were sown 0.5 cm deep on the 23 December 2010; pasture growth and development were monitored for a period of 26 months (Calvelo Pereira *et al.*, 2014; Calvelo Pereira *et al.*, 2016).

Soil fractionation procedure and C allocation to pools

At the end of the experiment (*i.e.*, after 26 months of pasture growth), the PVC pipe lysimeters were dismantled and fresh soil samples were taken for further fractionation. A total of 34 fresh soil samples were used: 17 topsoils (0–2 cm depth) and 17 subsoils (14–16 cm depth).

All fresh soil samples were gently crushed to pass a 2 mm sieve, discarding coarse root fragments. A subsample of each of the corresponding fresh soils obtained after 2-mm sieving

was dried until constant weight, thoroughly mixed, gently ground (< 250 μm) and stored. A ground aliquot of each individual dried sample obtained was used for total C determination using a vario MACRO cube CHNS elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

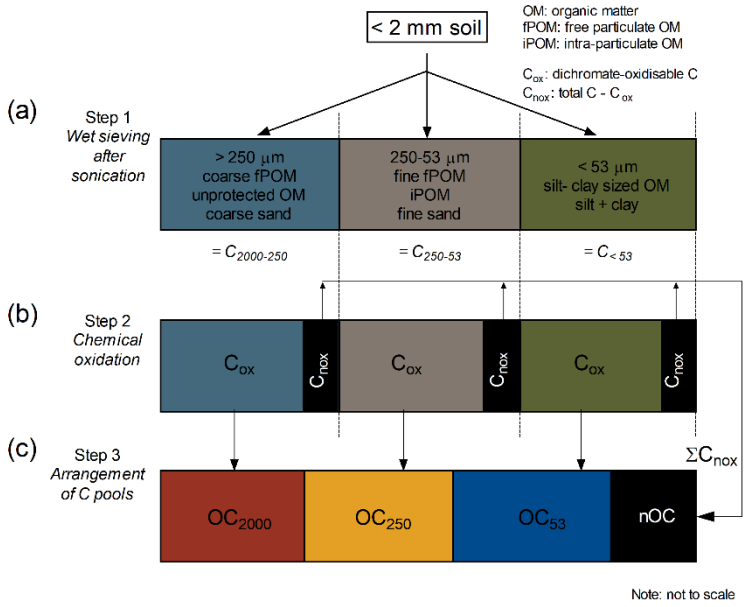

Figure 1 Soil fractionation procedure and C allocation to pools: (a) step 1, wet sieving after sonication; (b) step 2, chemical oxidation; and (c) step 3, arrangement of SOC pools. See text for a detailed description of the different steps.

Subsequently, all 2-mm sieved fresh soil samples were fractionated following a three-step procedure: (1) particle size fractionation or step 1; (2) chemical oxidation or step 2; and (3) virtual arrangement of SOC pools or step 3.

Step 1: particle size fractionation after sonication and wet sieving (Curtin et al., 2016)

Particle size separation of fresh soil samples (< 2 mm) was carried out after dispersing soil using an ultrasonic probe (approx. 10 g soil in 30mL deionised water; 60 s sonication; power output 64 J s^{-1}). Subsequently, wet sieving was used to separate the following fractions of different particle size (Figure 1a): (i) 2000–250 μm; (ii) 250–53 μm; and (iii) < 53 μm. The fraction above 250 μm (*i.e.*, 2000–250 μm) was separated by passing the dispersed soil suspension trough a 250 μm sieve and it is referred thereafter as 2000–250. This fraction corresponds to a coarse particulate organic matter (cPOM) fraction. The fraction below 250 μm was further sieved by passing the dispersed soil suspension through a 53 μm sieve to obtain two more separates: fraction 250–53 μm, referred as fine particulate organic matter (fPOM) and fraction < 53 μm, usually referred as the "fine fraction" (Figure 1a).

All samples for each of the corresponding particle-size fractions obtained were dried in oven (45 °C) until constant weight, thoroughly mixed, gently ground (< 250 μm) and stored. A ground aliquot of each individual sample obtained was used for total C determination using a vario MACRO cube CHNS elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany). C and N content is expressed per kg of dried soil unless otherwise stated. A second aliquot of each individual sample obtained in step 1 was used for further chemical fractionation (Step 2, below).

Step 2: chemical oxidation of individual fractions by using potassium dichromate

Ground soil samples obtained in step 1 were additionally treated with potassium dichromate ($K_2Cr_2O_7$) following Herath *et al.* (2014) and Calvelo Pereira *et al.* (2011) to determine the oxidisable organic C (C_{ox}). The non-oxidisable C (C_{nox}), i.e, the difference total C – C_{ox}, was calculated for each of the soil fractions obtained in step 1. C_{nox} corresponds to a sum of alkyl C and pyrogenic C that is not oxidised after mixing with a concentrated dichromate solution (Knicker *et al.*, 2007; Calvelo Pereira *et al.*, 2011; Suárez-Abelenda *et al.*, 2014).

Step 3: Virtual arrangement of SOC pools

The amount of C_{ox} obtained for each fraction in step 1 was considered as the organic C (OC) pool associated with a specific particle size, as follows: (i) OC in the 2000–250 μm particle size fraction (referred as OC_{2000}); (ii) OC in the 250–53 μm particle size fraction (referred as OC_{250}); and (iii) OC in the < 53 μm particle size fraction (referred as OC_{53}; Figure 1c). The final arrangement of C pools considered that the total amount of organic C resisting dichromate oxidation (referred thereafter as nOC; Figure 1c) was the result of summing all individual C_{nox} fractions calculated in step 2 for each soil sample.

Statistical analyses

Statistical analyses were conducted with Statistica version 8 software package (Stat Soft. Inc., Tulsa, OK, USA). Data were statistically analysed using the factorial ANOVA procedure. The model included the fixed effect of the pasture type (*i.e.*, ryegrass and red clover + cocksfoot), the type of amendment (*i.e.*, nil or non-biochar amended soil and biochar-amended soil), and the interaction of amendment and pasture type. If a significant ($P < 0.05$) main effect was detected, difference between treatment means was tested using the least significant difference.

Results and Discussion

Distribution of soil in particle size fractions

The distribution of the soil in the fractions considered in this study, at the end of the experiment, varied with the depth and soil pasture considered (Table 1). The amount of soil < 2 mm distributed in fractions of size 2000–250, 250–53, < 53 μm varied substantially, over average wide ranges of 0.01–0.03 g $soil_{2000–250}$/g $soil_{<2mm}$, 0.15–0.20 g $soil_{250–53}$/g $soil_{<2mm}$, and 0.76–0.84 g $soil_{< 53}$/g $soil_{<2mm}$, respectively (Table 1). Depth and pasture type explained the distribution of soil among the different fractions (Table 1). At 14–16 cm depth, the presence of biochar increased (at $P < 0.05$) the average mass of soil allocated to the 2000–250 μm fraction for both pastures considered (Table 1). In all cases ≥ 76% of the dry soil weight was found in the fraction of size <53 μm (Table 1). Recovery of the initial mass of soil after the fractionation was good, and varied among 98% and 100% (Table 1).

Table 1 Average (n = 4) changes in soil mass (g soil in fraction / g soil$_{<2\ mm}$) allocated per fraction and recovery (%) per depth (*i.e.* 0–2 cm and 14–16 cm) obtained 2 years after "cultivation", growth of different pasture species (*i.e.*, ryegrass and a mixture of red clover + cocksfoot) and biochar amendment (*i.e.* nil or non-biochar and biochar-amended) in lysimeters containing Tokomaru silt loam. Results (*P* value) from a factorial ANOVA analysis considering the main effect of Pasture, Amendment and the interaction between Pasture and Amendment are also included. Data at t = 0, corresponding to each sampling depth in the lysimeter at the beginning of the experiment, are included as reference.

Depth	Soil fraction (μm)		At t=0	Ryegrass		Mixture		SEM	*P* value		
				Nil	Biochar	Nil	Biochar		Pasture	Amendment	Pasture × Amendment
0–2 cm	2000–250	g/g	0.01	0.02	0.01	0.03	0.03	0.002	0.001	NS	NS
	250–53	g/g	0.13	0.15	0.15	0.18	0.18	0.004	0.005	NS	NS
	<53	g/g	0.85	0.81	0.82	0.78	0.78	0.006	0.001	NS	NS
Recovery		*%*	*98.8*	*97.8*	*99.2*	*98.6*	*98.8*	*0.4*	*NS*	*NS*	*NS*
14–16 cm	2000–250	g/g	0.02	0.01	0.02	0.02	0.02	0.002	0.016	0.023	NS
	250–53	g/g	0.23	0.15	0.17	0.20	0.20	0.008	0.002	NS	NS
	<53	g/g	0.76	0.84	0.80	0.77	0.76	0.011	0.007	NS	NS
Recovery		*%*	*100.0*	*99.5*	*98.6*	*98.8*	*98.9*	*0.2*	*NS*	*NS*	*NS*

Table 2 Average (n = 4) changes in soil total soil C (g C / kg soil$_{<2\ mm}$) and C (g C in fraction / kg soil$_{<2\ mm}$) allocated per fraction per depth (*i.e.* 0–2 cm and 14–16 cm) obtained 2 years after "cultivation", growth of different pasture species (*i.e.*,. ryegrass and a mixture of red clover + cocksfoot) and biochar amendment (*i.e.* nil or non-biochar and biochar-amended) in lysimeters containing Tokomaru silt loam. Results (*P* value) from a factorial ANOVA analysis considering the main effect of Pasture, Amendment and the interaction between Pasture and Amendment are also included. Data at t = 0, corresponding to each sampling depth in the lysimeter at the beginning of the experiment, are included as reference.

Depth	Soil fraction (μm)		At t=0	Ryegrass		Mixture		SEM	*P* value		
				Nil	Biochar	Nil	Biochar		Pasture	Amendment	Pasture × Amendment
0–2 cm	<2 mm	g/kg	19.9	23.7	23.5	30.9	31.2	1.1	<0.001	NS	NS
	2000–250	g/kg	0.5	2.1	2.2	6.1	6.6	0.7	<0.001	NS	NS
	250–53	g/kg	1.0	2.1	2.2	4.6	4.9	0.4	<0.001	NS	NS
	<53	g/kg	18.4	19.5	19.0	20.2	19.7	0.3	NS	NS	NS
14–16 cm	<2 mm	g/kg	36.3	23.4	32.4	30.1	39.5	1.6	<0.001	<0.001	NS
	2000–250	g/kg	3.0	0.9	4.8	2.7	8.2	0.8	0.020	<0.001	NS
	250–53	g/kg	7.6	1.8	4.5	6.9	8.9	0.8	<0.001	0.012	NS
	<53	g/kg	25.8	20.8	23.1	20.5	22.4	0.7	NS	NS	NS

Allocation of soil C to fractions of size 2000–250, 250–53, and < 53 μm

Average total C concentration of the samples used was variable, ranging between 23.4 and 34.5 g C/kg soil$_{<2mm}$ (Table 2). Concentration of C and N in the fractions obtained was also very variable, especially considering the soil depth and pasture type, as well as biochar amendment (at 14–16 cm depth only, as expected) (Table 2). In order to assess the distribution of C between the different fractions defined after sonication and wet sieving only, the concentration of C was expressed per kg of bulk soil < 2mm. Concentration of C in 2000–250, 250–53, and < 53 μm fractions varied substantially, over ranges of 0.5–10.4 g $C_{2000–250}$/kg soil$_{<2mm}$, 1.0–11.8 g $C_{250–53}$/kg soil$_{<2mm}$, and 15.5–25.8 g $C_{<53}$ / kg soil$_{<2mm}$ respectively (Table 2). For each pasture type, both the 0–2 cm depth and the 14–16 cm depth showed similar amounts of C. On average, the loss of some soil after the fractionation procedure applied (Table 1) also implied a loss of C. In fact, the recovery of C [*i.e.*, ($C_{2000–250}$ + $C_{250–53}$ + $C_{< 53}$ / total $C_{<2mm}$) × 100]) varied over ranges of 80–112% for C (average: 100%) (data not shown). As expected, the fraction < 53 μm contained most of the C determined (on average, 73% of total C recovered). This is consistent with previous work, which shows that > 70% of soil C is associated with the fine fraction and hence regarded as the stabilised C (Gregorich *et al.*, 2006; McNally *et al.*, 2017).

The type of swards influenced the distribution of C in the 2000–250 and 250–53 μm fractions and the mixture tended to allocate more C in those fractions than ryegrass (Table 2). The application of pine biochar increased the amount of C detected in the fractions of size 2000–250 and 250–53 μm in the 14–16 cm depth, as expected. However, the C concentration detected at each depth in the fraction of size < 53 μm was independent of the pasture type and soil amendment (Table 2).

Allocation of soil C to SOC pools

Each of the fractions obtained after sonication and wet sieving (nominally of size 2000–250, 250–53, < 53 μm) was chemically treated with potassium dichromate, which allowed the differentiation of two sub-fractions: (i) the oxidisable-C (C_{ox}); and (ii) the non-oxidisable or resistant C (C_{nox}) (Figure 1b). As expected, most of the organic C in each of the soil fractions was oxidisable, and the concentration of organic C varied substantially, with ranges of 0.5–8.6 g OC_{2000}/kg soil$_{<2mm}$, 0.9–9.0 g OC_{250}/kg soil$_{<2mm}$, and 14.7–24.4 g $OC_{<53}$/kg soil$_{<2mm}$ (Figure 2). The amount of C in the resistant fraction was also variable, with values ranging between 0.2 and 12.4 g nOC/kg soil$_{<2mm}$ (Figure 2). When expressed as percentage of the total soil C obtained (*i.e.*, g OC_{2000}, OC_{250}, OC_{53} or nOC /100 g total C recovered), up to 67% of the soil C was associated to the OC_{53} pool, whereas only the 10% of the C was found in the OC_{2000} pool (data not shown).

The type of swards influenced the distribution of C in the different C pools, as the growth of mixture tended to allocate more C in the OC_{2000} and OC_{250} pools than ryegrass at both depths studied (Figure 2). On the other hand, the organic C concentration detected at each depth in the OC_{53} pool was independent of the pasture type and soil amendment (Figure 2). The application of pine biochar increased the amount of C detected in the OC_{2000} and nOC pools, in the 14–16 cm depth (Figure 2). This finding, if validated by a direct measure of native SOC and pyrogenic C, would suggest that pine biochar favoured the accumulation of particulate organic matter (or POC; see below) in the subsoil 2 years after "cultivation" and growth of different pasture species, despite the lack of influence of biochar amendment on annual herbage production (Calvelo Pereira *et al.*, 2016).

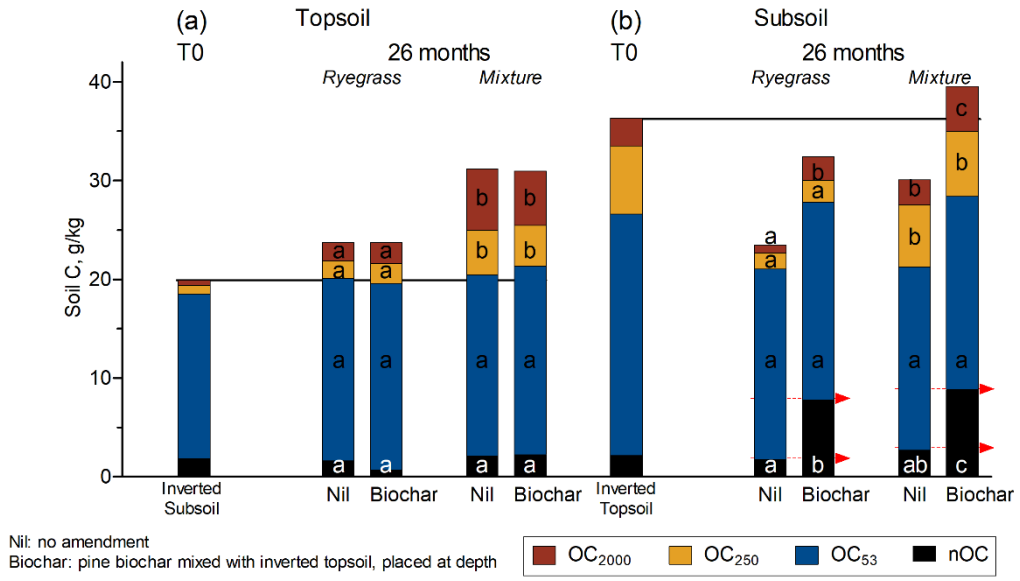

Figure 2 Average C concentration (g C in each fraction /kg soil$_{<2mm}$; n = 4) in (a) the 0–2 cm depth layer (topsoil), and (b) the 14–16 cm depth layer (subsoil) allocated to the different SOC pools considered: OC_{2000}, OC_{250}, OC_{53} and nOC obtained 2 years after "cultivation", growth of different pasture species (i.e,. ryegrass and a mixture of red clover + cocksfoot) and biochar amendment (*i.e.*, nil or non-biochar and biochar-amended) in lysimeters containing Tokomaru silt loam. For each pool and depth, different letters indicate differences ($P < 0.05$) between average values as obtained from a factorial ANOVA analysis considering the main effect of Pasture, Amendment and the interaction between Pasture and Amendment. Values at t = 0 (T0), corresponding to each sampling depth in the lysimeter at the beginning of the experiment, are included as reference; the horizontal black line indicates level total C concentration at T0. Horizontal dotted lines indicate the relative size of the nOC pool corresponding to biochar addition (see text for details).

The net difference between $nOC_{Biochar} - nOC_{Nil}$, 26 months after cultivation and plant growth was 6.0 g C/kg soil$_{<2mm}$ under ryegrass and 6.2 g C/kg soil$_{<2mm}$ under the mixture of red clover + cocksfoot (Figure 2). Thus, the apparent net amount of resistant C is in the order of the actual amount of biochar C added at the beginning of the experiment, or approx. 6.48 g $C_{biochar}$/kg soil (based on bulk density, 1.17 kg/m^3, 10 cm depth, 10 tonnes biochar/ha; 759 g C/kg biochar). This value is indicative only, as the current method does not determine directly the amount of pyrogenic C.

Taken together, OC_{2000} and OC_{250} pools resemble the POC pool that is commonly referred in the literature (Gregorich *et al.*, 2006; Baldock *et al.*, 2013b; Poeplau *et al.*, 2013), because of the particle size and the susceptibility to chemical oxidation of the organic C present. In addition, the OC_{53} pool would be equivalent to the HOC pool, whereas the nOC pool is, by definition, similar to the ROC pool (Skjemstad *et al.*, 2004). Future research needs to address the correct validation of these equivalences by studying the C fractionation using other techniques (*e.g.*, spectroscopy; Baldock *et al.* (2013a), Viscarra Rossel *et al.* (2017)).

Conclusions

We found that the combined use of simple wet chemistry methods as dichromate oxidation, as well as sonication and wet sieving, is suitable for allocating soil C to measurable SOC pools. The methodology allowed the virtual separation of a resistant C fraction that resembles that of the pyrogenic C added at the beginning of the experiment to the total SOC pool. The application of the described methodology to other soil types with and without pyrogenic C sources will contribute to the optimisation of the procedure; in the end, a correct standardisation of the methodology would include an inter-laboratory comparison. We propose that the fractions obtained by using this methodology, after further optimisation and standardisation, may be comparable to the POC, HOC and ROC pools used in models (*e.g.*, RothC). Future research is needed to investigate this correspondence in detail.

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