Pathways of litter C by formation of aggregates and SOM density fractions: Implications from ^{13}C natural abundance

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ABSTRACT

Aggregate formation is a key process of soil development, which promotes carbon (C) stabilization by hindering decomposition of particulate organic matter (POM) and its interactions with mineral particles. C stabilization processes lead to ^{13}C fractionation and consequently to various δ^{13}C values of soil organic matter (SOM) fractions. Differences in δ^{13}C within the aggregates and fractions may have two reasons: 1) preferential stabilization of organic compounds with light or heavy δ^{13}C and/or 2) stabilization of organic materials after passing one or more microbial utilization cycles, leading to heavier δ^{13}C in remaining C. We hypothesized that: 1) ^{13}C enrichment between the SOM fractions corresponds to successive steps of SOM formation; 2) ^{13}C fractionation (but not the δ^{13}C signature) depends mainly on the transformation steps and not on the C precursors. Consequently, minimal differences between Δ^{13}C of SOM fractions between various ecosystems correspond to maximal probability of the SOM formation pathways.

We tested these hypotheses on three soils formed from cover loam during 45 years of growth of coniferous or deciduous forests or arable crops. Organic C pools in large macroaggregates, small macroaggregates, and microaggregates were fractionated sequentially for four density fractions to obtain free POM with $\rho < 1.6$ g cm^{-3}, occluded POM with two densities ($\rho < 1.6$ and 1.6–2.0 g cm^{-3}), and mineral fraction ($\rho > 2.0$ g cm^{-3}).

The density fractions were ^{13}C enriched in the order: free POM < light occluded POM < heavy occluded POM < mineral fraction. This, as well as their C/N ratios confirmed that free POM was close to initial plant material, whereas the mineral fraction was the most microbially processed. To evaluate the successive steps of SOM formation, the Δ^{13}C values between δ^{13}C of SOM fractions and δ^{13}C of bulk SOM were calculated. The Δ^{13}C indicated that, parallel with biochemical transformations, the physical disintegration strongly contributed to the formation of free and occluded light POM. In contrast, biochemical transformations were more important than physical disintegration for formation of heavy occluded POM from light occluded POM. This was confirmed by review of 70 Δ^{13}C values from other studies analyzed Δ^{13}C depending on the density of SOM fractions. Accordingly, the successive steps of SOM formation were: fLF$_{<1.6}$ = oLF$_{<1.6}$ → oDF$_{1.6–2.0}$ = MF$_{>2.0}$. The obtained steps of C stabilization were independent on the initial precursors (litter of coniferous forest, deciduous forest or grasses).

To test the second hypothesis, we proposed an extended scheme of C flows between the 3 aggregate size classes and 4 SOM fractions. Δ^{13}C enrichment of the SOM fractions showed that the main direction of C flows within the aggregates and SOM fractions was from the macroaggregate-free POM to the mineral microaggregate fraction. This confirmed the earlier concept of SOM turnover in aggregates, but for the first time quantified the C flows within the aggregates and SOM density fractions based on δ^{13}C values. So, the new ^{13}C natural abundance approach is suitable for analysis of C pathways by SOM formation under *steady state* without ^{13}C or ^{14}C labeling.

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1. Introduction

Aggregate formation is one of the main soil-forming processes, distinguishing soils from their parent materials. Plant residues and

root exudates are assumed to be the main drivers of aggregation in the most models of soil structure development (Tisdall and Oades, 1982; Oades, 1984; Six et al., 1999). Nonetheless, the general theoretical principles and hierarchy of aggregate formation were seldom tested experimentally (Angers et al., 1997), mainly because suitable approaches were limited.

Only few methods enable determining the formation of aggregate size classes and the matter exchange between them: microscopic observation of previously disrupted aggregates; labeling of aggregates with radionuclides such as ^{60}Co (Toth and Alderfer, 1960), ^{56}Fe (Wooldridge, 1965) or with the rare earth element oxides La$_2$O$_3$, Pr$_6$O$_{11}$, Nd$_2$O$_3$, Sm$_2$O$_3$, and Gd$_2$O$_3$ (Zhang et al., 2001); and application of ^{13}C and ^{14}C isotopes (Majumder and Kuzyakov, 2010).

Transmission electron microscopy has been used to analyze the aggregate formation starting from individual particles to macro-aggregates (Tisdall and Oades, 1982). Although, this approach distinguishes the particle components and their size, it does not explain mechanisms of aggregate formation or matter exchange between the size classes.

Labeling aggregates with radionuclides, mentioned above, or with ceramic/glass spheres enabled tracing the translocation of particles within and between aggregate size classes (Plante et al., 1999) and to investigate aggregate dynamics (Plante et al., 2002; Plante and McGill, 2002). Accordingly, the mean residence time of macroaggregates ranged from 5 to 33 days (Plante and McGill, 2002). These methods however, are limited for mineral particles and do not allow analyzing the role of aggregates in C stabilization.

To link the aggregate dynamics with the organic substances, ^{13}C and ^{14}C isotopes have been applied. For example, the primary formation of macroaggregates around fresh plant residues was proved using ^{13}C labeled wheat straw (Angers et al., 1997). Moreover, during long-term incubation of ^{13}C labeled plant residues in soil, the ^{13}C enrichment of macroaggregates decreased, whereas microaggregates increased (Angers et al., 1997). These results, however, focused on the transformation of organic substances during aggregate formation and do not explain aggregate formation *per se*. Thus, even though information on the sequence of aggregate formation and aggregate turnover exists, no clear evidence is available about what the sources are and what the products of organic materials in various aggregate size classes are.

Additional difficulties in studying aggregate formation and its role in C stabilization arise due to the heterogeneity of soil organic matter (SOM) in aggregate size classes (Besnard et al., 1996; Six et al., 1998; Yamashita et al., 2006). Free particulate organic matter (fPOM), light and heavy occluded POM (oPOM), and mineral-associated organic matter (OM) have been distinguished from the aggregates by physical (Yamashita et al., 2006) and chemical fractionation methods (Stewart et al., 2008). This means that aggregates can include SOM of various origin, composition and degree of microbial degradation, i.e. as "products" or as "sources". The quality of SOM fractions has been analyzed based on the C/N ratios and NMR spectroscopy. These approaches have shown the following order of SOM fraction formation: free POM → heavy occluded POM → light occluded POM → mineral fraction (Golchin et al., 1998). Based on δ^{13}C analysis, however, the following formation sequence has been proposed: free POM → light occluded POM → heavy occluded POM → mineral fraction (Baisden and Amundson, 2002). Moreover, the simultaneous presence of light occluded and heavy occluded POM as one pool has been noted based on ^{14}C dating (Baisden and Amundson, 2002). There is therefore no unitary concept of SOM fraction formation and especially of the C flows between the SOM fractions separated from the aggregate size classes.

A promising approach to studying the sources of organic materials and C flows within and between the aggregates is using natural differences in the stable isotope composition of aggregates and SOM fractions. The differences in stable C isotope composition (^{13}C/^{12}C) may have two reasons (Werth and Kuzyakov, 2010): 1) preferential stabilization of substrates with light or heavy δ^{13}C and/ or 2) stabilization of organic materials after passing one or more microbial utilization cycles, leading to heavier δ^{13}C in remaining organic matter (because of release of CO$_2$ with light δ^{13}C). The first mechanism − stabilization of preferential substrates with light or heavy δ^{13}C − would occur mainly by chemical sorption of specific groups of organic substances, e.g. with light (lipids, phenols, lignins) or heavy δ^{13}C (cellulose, amino acids, hemicellulose) (Sollins et al., 2006; von Luetzow et al., 2006). This would lead to divergent δ^{13}C signatures in various aggregate size classes and density fractions and would not be connected with steps of organic C utilization by microorganisms. Importantly, however, microbial uptake and utilization of organic materials outcompete all physicochemical processes in soil (Fischer et al., 2010). Therefore, we assume the dominance of the second mechanism − stabilization of organic materials after passing one or more microbial utilization cycles. In contrast to the first mechanism, the second one generally leads to heavier δ^{13}C in remaining organic matter in soil. This is connected with the preferential decomposition of light C to CO$_2$ and remaining heavier C in microorganisms and their residues (Werth and Kuzyakov, 2010) that are a key SOM source (Kindler et al., 2006; Miltner et al., 2012). The δ^{13}C increase by this mechanism is proportional to the number of cycles in which C is passed through the microorganisms before being stabilized in SOM.

We combined aggregate's size and density fractionations with natural differences in stable C isotope composition to investigate possible flows of C between and within the aggregate size classes. Using the δ^{13}C approach, we hypothesized: 1) During SOM formation, the C remaining in soil by litter decomposition will be enriched by ^{13}C (as the light C will be evolved as CO$_2$). This leads to a δ^{13}C increase both: of total SOM compared to plant residues, as well as of SOM fractions formed within individual steps of SOM formation. Consequently, the ^{13}C enrichment (=δ^{13}C less negative) of one SOM fraction compared to the other shows that the more enriched is the product of the less enriched one. Based on these ^{13}C enrichments between the fractions, it is possible to predict the formation steps of SOM fractions. 2) ^{13}C fractionation within individual steps of the formation of SOM pools and fractions is relatively constant, independent of the ecosystem type. Thus, the ^{13}C fractionation *per se* (Δ^{13}C, but not the δ^{13}C of the SOM fraction) depends mainly on the transformation steps of the fraction but not on the δ^{13}C of the precursors. This is true for continuous C input. Consequently, minimal differences between Δ^{13}C of SOM fractions in various ecosystems correspond to the maximal probability of the SOM formation pathways.

2. Materials and methods

2.1. Experimental set-up and soil sampling

Soils from large open lysimeters located within the Moscow State Lomonosov-University area were used. The installation of the lysimeters has been described in detail elsewhere (Vinnik and Bolyshev, 1972). Briefly, the large lysimeters were installed in 1965 and included 20 plots of 3 × 3 m (area 9 m^2) and depth of 1.5 m. Each lysimeter was filled with carbonate-free loam, which originated from the cover loams of Valday (corresponds to Würm/ Weichsel/Wisconsin) glaciation, collected in the north of the Moscow region. The texture of this parent material was silty loam with the following particle distribution: 3.65% sand, 65.3% silt, and 28.6% clay (Vinnik and Bolyshev, 1972). The predominant minerals of the clay fraction were smectites (56.1%), illites (34.0%), and

kaolinites with chlorites (8.9%) (Chizhikova et al., 2006b). This parent material was sieved through a 4 cm mesh size and thoroughly mixed prior to filling. The chemical characteristics of the initial parent material and the soil − haplic Regosol siltic clayic − that developed during the 45 years are presented in Table 1.

After two years of settling, the following plant communities were planted: coniferous forest, deciduous forest (four plots for each type of forest) and arable/grassland (two plots). Only the plant communities that are described here were used in this study; other plant communities were presented earlier (Vinnik and Bolyshev, 1972; Gerasimova et al., 1989). Sixty-one 5-year-old seedlings of Norway spruce (*Picea abies*) were planted in each lysimeter with coniferous forest; 31 English oak (*Quercus robur*) seedlings and 30 3-year-old seedlings of Norway maple (*Acer platanoides)* were planted in each deciduous forest plot. The arable/grassland plots included agricultural nine crop rotation, among them potato, summer wheat, buckwheat, oat, barley, and perennial grasses. Crop rotation was conducted during 35 years, during which the soil was manually plowed; in the last 10 years, permanent grasses were grown. There were no inputs of organic fertilizers like manure and no C_4 plants were grown on the plots.

The climate of the Moscow region is humid continental. The mean annual temperature in the area of the Moscow State University is $+5.8$ °C, with July and February means of $+19.2$ °C and -6.7 °C, respectively. The mean precipitation is 680 mm.

Soil samples (200 g) from the lysimeters were taken in spring 2012 after 45 years of plant community development on the initial parent material, from a depth of 0−5 cm, with four field replicates for each plant community. Four field replicates means that one soil sample was collected from the each of four plots for each vegetation type. In the arable/grassland plot the two samples were taken from the two available plots. We will use the following terms for the three soils: "coniferous forest", "deciduous forest" for mixed broadleaf forest, and "arable land".

The soils were sampled from the 0−5 cm layer because the greatest differences in organic C content were observed in this soil layer (Saveliev and Vladychenskii, 2001). Moreover, only the first 20 cm of initial parent material were changed by soil formation, as determined based on the color of the soil profile and chemical analyses (C contents, pH, changes) (Chizhikova et al., 2006a; Verkhovets et al., 2006; Vinnik and Bolyshev, 1972). The upper 0−5 cm soil layer reacts first to plant community development, whereas the lower horizons react much slower (Vesterdal et al., 2008).

2.2. Aggregate size fractionation

The soil samples (100 g each) were air-dried at room temperature and sieved through the 2000 and 250 μm meshes on the Vibratory Sieve Shaker AS 200 (Retsch, Germany) for 5 min, amplitude 1.5 mm. Large macroaggregates (>2000 μm), small macroaggregates (250−2000 μm), and microaggregates (<250 μm) were obtained. Dry sieving was used instead of wet sieving 1) to minimize the disruption of aggregates, and consequently 2) to avoid redistribution of fine organic particles from large and medium to microaggregate size class, and 3) to prevent the leaching of dissolved organic matter to the microaggregate size class.

2.3. Separation by density fractionation

The density fractions were separated for each of the three aggregate size classes obtained by the dry sieving described above. To isolate the density fractions, the method of John et al. (2005), as modified by Dorodnikov et al. (2011), was applied. In total, four fractions were obtained from each aggregate size. Three grams of air-dried aggregates were placed into a centrifugation tube and 15 ml of sodium polytungstate (SPT) solution with a density of 1.6 g cm^{-3} was added. After the tube was gently inverted several times, the solution was centrifuged at 4000 rpm for 1 h and the supernatant with floating particles was filtered (cellulose acetate filter, 0.45 μm; Sartorius, Germany) and washed with distilled water to obtain a *free light fraction* of OM with $\rho < 1.6$ g cm^{-3} (fLF$_{<1.6}$). The remaining soil was shaken for 16 h (60 movements per minute) with 10 ml of SPT solution ($\rho = 1.6$ g cm^{-3}) and 5 glass beads ($d = 5$ mm) to crush the aggregates. After this, the soil suspension was centrifuged for 1 h at 4000 rpm. The supernatant was filtered (as for the first fraction), washed with distilled water to gain an *occluded light fraction* of OM with $\rho < 1.6$ g cm^{-3} (oLF$_{<1.6}$). The soil residue was dispersed with 10 ml of SPT solution $\rho = 2.0$ g cm^{-3}, centrifuged for 1 h at 4000 rpm, and the supernatant was filtered and washed as the previous two steps to obtain an *occluded dense fraction* of OM with $\rho = 1.6$−2.0 g cm^{-3} (oDF$_{1.6−2.0}$). The soil remaining after the separation of the light fractions was washed four times with distilled water (20 ml each time) to generate the *mineral fraction* with $\rho > 2.0$ g cm^{-3} (MF$_{>2.0}$).

To better present the data in graphs, the average density of fLF$_{<1.6}$ is accepted as 1.2 g cm^{-3}, because the usually reported density of plant residues is in the range 1.1−1.2 (Rühlmann et al., 2006). The density of the oLF$_{<1.6}$ is accepted as 1.4 g cm^{-3}; the density for oDF$_{1.6−2.0}$ is 1.8 g cm^{-3} (the middle between 1.6 and 2.0 g cm^{-3}), and the density for the MF$_{>2.0}$ is 2.2 g cm^{-3}.

2.4. Analysis of C content and $\delta^{13}C$ values

All aggregate size classes and fractions obtained by density fractionation were dried at 40 °C, weighed, and ground (MM2, Fa Retsch, Germany) before analysis. The C and N contents of bulk soils, aggregate size classes, and isolated density fractions were measured using an elemental analyzer (Vario EL II, Germany).

The natural stable carbon-isotope ratio of all fractions and of bulk soil samples and plant litters were measured at the Centre for Stable Isotope Research and Analysis (KOSI), University of Goettingen, using an elemental analyzer in a dual-element analysis mode (Carlo Erba 1108, Milano, Italy). The C isotope ratios were expressed relative to the international PDB limestone standard as $\delta^{13}C$.

2.5. Calculations and statistics

Statistical analyses were performed using Statistica 7.0 software. The effect of plant community on aggregate composition, the distribution of SOC and C/N ratio in aggregate size classes were tested by ANOVA followed by the Tukey HSD post-hoc test (significance level $p < 0.05$). Significant differences between $\delta^{13}C$ of SOM fractions were tested with the Tukey HSD post-hoc test (significance level $p < 0.05$). All data are presented as four replicates ± standard

Table 1
pH, soil organic carbon (SOC), total nitrogen (TN), and C/N ratio of soils (0−5 cm depth) in the large lysimeters after 45 years (sampled in 2012) development under three vegetation types on the cover loam (initial parent material).

Properties	Cover loam[a]	Coniferous forest	Deciduous forest	Arable land
pH	5.7 ± 0.1	5.4 ± 0.1	6.4 ± 0.1	6.2 ± 0.1
SOC (g kg^{-1} soil)	0.7	29.6 ± 2.3	43.1 ± 4.0	11.6 ± 0.7
TN (g kg^{-1} soil)	0.3	1.2 ± 0.2	3.1 ± 0.5	0.9 ± 0.1
C/N	2.3	26.1 ± 1.3	21.0 ± 1.5	16.5 ± 1.7

[a] The data on initial parent material were taken from Gerasimova et al. (1989).

Table 2
Aggregate composition [%], C content [g kg^{-1}], and C/N ratios in dry sieved aggregates in soils under coniferous, deciduous forests, and arable land. Letters reflect significant differences between the soils, whereas stars reflect significant differences between aggregate size classes within one soil.

Aggregate size (μm)	Aggregate composition (%)	C (g kg^{-1})	C/N
Coniferous forest			
>2000	64.6 (3.9)b	18.3 (3.3)a	26.1 (1.1)a
250−2000	28.9 (3.2)a	42.1 (9.9)ab	28.7 (1.4)a
<250	5.9 (0.4)a*	32.4 (2.7)b	32.1 (1.1)a
Deciduous forest			
>2000	50.3 (3.8)ab*	35.2 (8.5)a	23.0 (0.9)a
250−2000	40.2 (3.3)a*	60.8 (11.5)a	23.4 (1.7)a
<250	9.6 (0.5)b*	49.0 (2.5)a	22.8 (1.9)b
Arable land			
>2000	78.9 (1.3)a*	11.8 (1.9)a	14.5 (0.2)b*
250−2000	15.1 (1.4)b*	10.4 (0.9)b	14.1 (0.4)b*
<250	5.9 (0.5)b*	12.6 (1.2)c	17.7 (1.1)b*

error. Regression lines were plotted through the mean values for the fractions of distinct density, separately for each soil.

3. Results

3.1. Carbon distribution and C/N ratios in aggregates and in density fractions

Large macroaggregates were the most abundant size class in all investigated soils (Table 2). The C content of aggregates did not differ between the size classes within investigated soils, but the differences were observed between the plant communities. The C/N ratio of the microaggregates in arable land soil was significantly higher than for other aggregate size classes (Table 2).
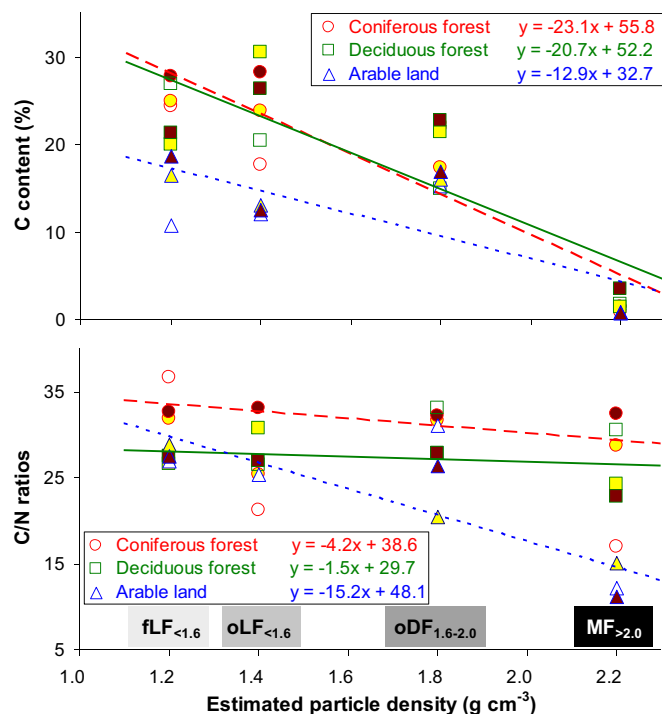

Fig. 1. C [%] (top) and C/N ratios (bottom) plotted vs. estimated particle density for the SOM fractions separated from the aggregate size classes from soils developed under coniferous (green circles), deciduous forests (purple squares), and arable land (brown triangles). The empty, yellow and brown symbols correspond to SOM fractions separated from large macroaggregates, small macroaggregates, and microaggregates, respectively. Values present mean of four replications. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The C content in fLF$_{<1.6}$ and oLF$_{<1.6}$ varied between 10 and 31% (Fig. 1 top). The C content in oDF$_{1.6−2.0}$ varied between 15 and 23% and was in a range of 0.5−4% in MF$_{>2.0}$. The C/N ratios of density fractions tended to decrease with increasing density (Fig. 1 bottom), showing the progressive degradation of plant C in the direction from fLF$_{<1.6}$ to MF$_{>2.0}$.

3.2. δ^{13}C values in aggregates, plant litter, and density fractions

The most negative δ^{13}C values of bulk SOM were observed in the arable soil, whereas maximal δ^{13}C values were obtained in coniferous forest soil (Fig. 2 top). The δ^{13}C values of the vegetation had the opposite trend. The δ^{13}C values of aggregates slightly decreased with decreasing size class (Fig. 2 top). The maximal difference between δ^{13}C of bulk SOM and initial plant residues was for the coniferous forest soil, the minimal − for arable land reflecting the physical distance between the main C input (O vs. Ah horizon) and consequently of microbial processing of C by stabilization.

The fLF$_{<1.6}$ and oLF$_{<1.6}$ in forest soils had similar δ^{13}C values, pointing to a similar degree of microbial processing of C in these fractions (Fig. 2 bottom). oDF$_{1.6−2.0}$ had lower δ^{13}C values compared to MF$_{>2.0}$ in forest soils. MF$_{>2.0}$ had the highest δ^{13}C, whereas fLF$_{<1.6}$ and oLF$_{<1.6}$ had the lowest δ^{13}C values in all aggregate size classes in the forest soils. This pattern was not strongly pronounced in arable soil (Fig. 2 bottom).

3.3. Changes in isotopic composition of SOM density fractions

To standardize the C isotopic composition in density fractions, the difference (Δ^{13}C) between δ^{13}C values of density fractions and δ^{13}C of the bulk SOM was calculated. The δ^{13}C values of the bulk SOM (and not δ^{13}C of plant litter) were subtracted from δ^{13}C of density fractions because the transformation of litter to SOM differs in the three vegetation types (Fig 2, top). These differences reflected the various quality of plant residues in the three ecosystems as well as different degrees of their decomposition relative to bulk SOM. This leads to large differences between the δ^{13}C of plant litter and δ^{13}C of SOM in the three ecosystems (Fig. 2 top).

The Δ^{13}C values were plotted against density of SOM fractions, and the regression lines were calculated for the each soil (Fig. 3). Calculated Δ^{13}C values of SOM fractions showed that the ^{13}C enrichment of SOM fractions increased on average by 2‰ with increasing density from 1.2 to 2.2 g cm^{-3} (Fig. 3). However, the ^{13}C was mainly enriched between 1.4 and 1.8 g cm^{-3}. The two light density fractions ($\rho \sim$ <1.2 and <1.4 g cm^{-3}) had similar Δ^{13}C values, pointing to similar degrees of decomposition. The density fractions 1.8 and 2.2 g cm^{-3} had close Δ^{13}C values and were more positive than the two lighter fractions. We therefore concluded that the two light fractions (fLF$_{<1.6}$ and oLF$_{<1.6}$) were products mainly of physical litter transformations (mechanical breakdown) and were not strongly subjected to microbial processing. In contrast, the two heaviest fractions were the products of stronger or more frequent microbial transformations.

3.4. Principal concept of C flows in the system of aggregates and density fractions

To analyze the possible formation pathways of SOM we arranged the fractions, separated from aggregate size classes, in the scheme consisting of three lines: upper line − macroaggregates, middle line − small macroaggregates and lower line − microaggregates (Fig. 4). Each line consists of four fractions arranged by increasing density: fLF$_{<1.6}$ − oLF$_{<1.6}$ − oDF$_{1.6−2.0}$ − MF$_{>2.0}$. Such an order assumes that increasing density leads to increasing transformation of plant C (Sollins et al., 2009; Dorodnikov et al., 2011).
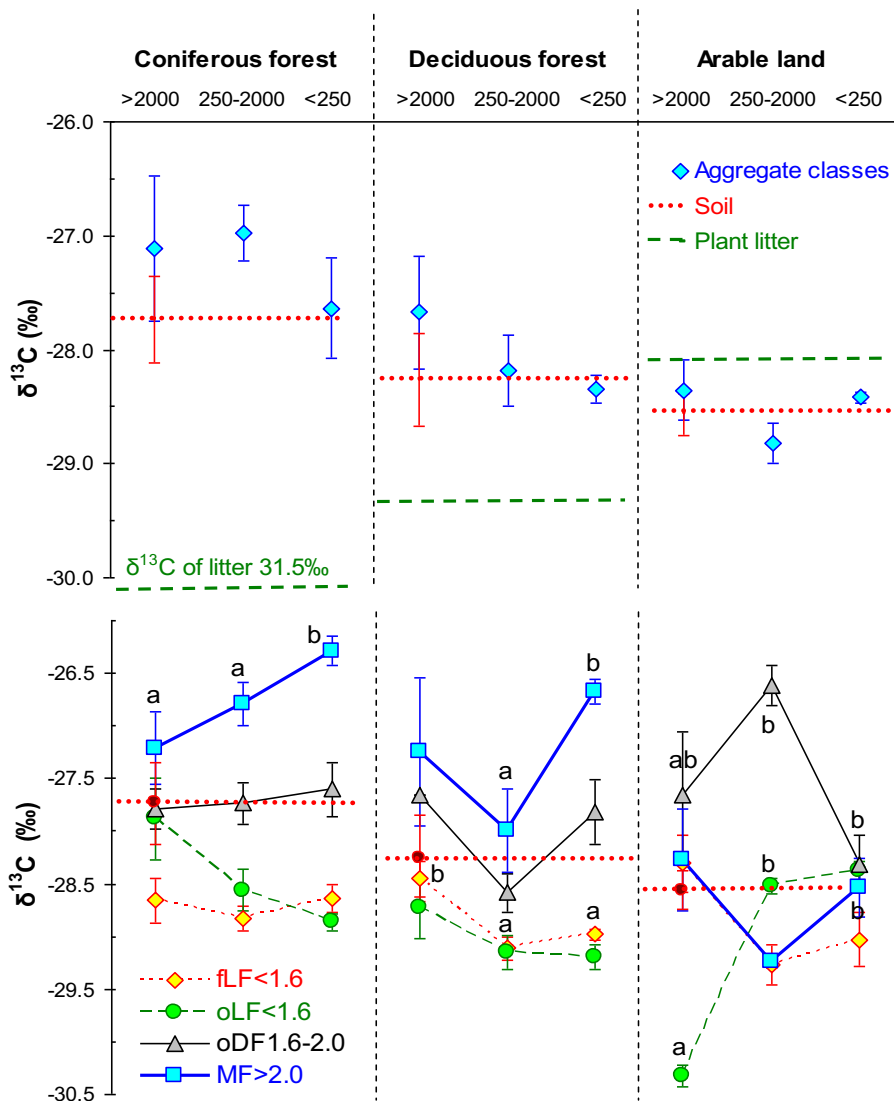
Fig. 2. $\delta^{13}C$ values of soil, plant litter, and aggregate size classes (top) and of SOM fractions (bottom) separated from the soils under coniferous, deciduous forests, and arable land. Values present mean of four replications ± standard error. Letters reflect significant differences for the fraction, separated from various aggregate size classes.

Thus, $fLF_{<1.6}$ was the starting point of the plant C flow into aggregates and $MF_{>2.0}$ was the latest stage of transformation. Near each node, we placed the $\Delta^{13}C$ values of density fractions separately for the coniferous forest, deciduous forest and arable land.

Considering that the increasing $\delta^{13}C$ in the fraction corresponds to the degree of its microbial transformation (Werth and Kuzyakov, 2010), we directed the arrows within as well as between the aggregate size classes in the scheme according to increasing $\Delta^{13}C$ values. Accordingly, the fractions at the start of the arrow were considered as "source" and at the end as "product". Thereafter, the $\Delta^{13}C$ of the "product" was subtracted from the $\Delta^{13}C$ of the "source" for the each soil (Supplementary Fig. 1). These three values were compared and the maximal difference between them was calculated. According to hypothesis 2, the closer the differences between the three ecosystems, i.e. the smaller the maximal difference, is the higher the probability of C pathways. Thus, arrow size in Fig. 4 corresponded to the similarities of the three ecosystems and, so, to the probability of ongoing C fluxes between the fractions.

Many studies (Baisden and Amundson, 2002; Sollins et al., 2009; Werth and Kuzyakov, 2010) showed that microbial transformation leads to increasing $\delta^{13}C$ value of the remaining organic C. Scheme

(Fig. 4) allows the conclusion that the main increase of $\Delta^{13}C$ values and consequently the direction of C flows within the aggregate classes are from free POM to the mineral fraction (from left to right in the scheme). The most probable C transformation occurs on the levels of small macroaggregates and microaggregates. However, C transformations can occur within one SOM fraction between the macro and microaggregates, as well as between SOM fractions separated from various aggregate size classes.

The starting point of the C flow on each level of aggregate size classes is $fLF_{<1.6}$ having the lightest $\Delta^{13}C$. The most probable C flow is from $fLF_{<1.6}$ of the small macroaggregates to $fLF_{<1.6}$ of microaggregates because the differences between the $\Delta^{13}C$ values of the three ecosystems are the lowest (0). The incorporation of C is highly probable from $fLF_{<1.6}$ to $oLF_{<1.6}$ for the small macro and microaggregates because the calculated differences between the $\Delta^{13}C$ values of the three ecosystems is between 0.7 and 0.8. According scheme (Fig. 4), C goes further from $oLF_{<1.6}$ to $oDF_{1.6-2.0}$, which corresponds to more positive $\Delta^{13}C$ of $oDF_{1.6-2.0}$ than of $oLF_{<1.6}$.

Carbon from $oDF_{1.6-2.0}$ of microaggregates is incorporated to $MF_{>2.0}$ of the large macroaggregates. Remarkably, the flow of heavy occluded C ($oDF_{1.6-2.0}$) from macroaggregates to $MF_{>2.0}$ is not very

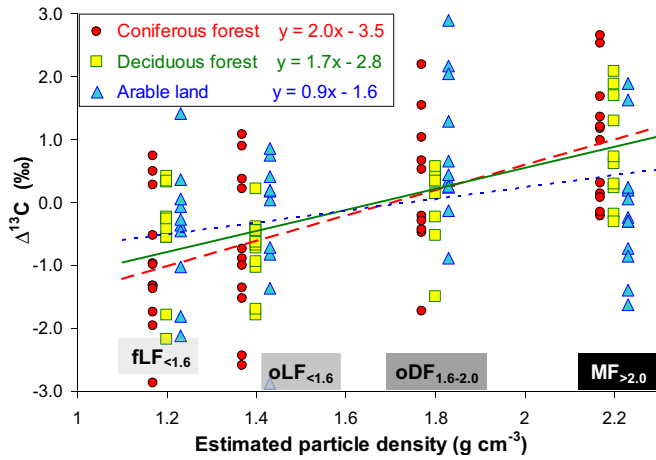**Fig. 3.** $\Delta^{13}C$ values of SOM density fractions (relative to $\delta^{13}C$ of bulk SOM) in respective soils plotted vs. estimated particle density of the SOM fractions, separated from aggregates in soils under coniferous, deciduous forests, and arable land. The regression lines represent the trends in the changes of $\Delta^{13}C$ values of SOM fractions for the each soil. The $\Delta^{13}C$ values of individual replications are presented. The $\Delta^{13}C$ values were shifted along the x-axis for the better presentation.

probable because the calculated differences between the $\Delta^{13}C$ values of the three ecosystems are very high (2.4–3.9). At the same time, C incorporation from microaggregate $oDF_{1.6-2.0}$ to $MF_{>2.0}$ of macroaggregates is more probable (values between 1 and 1.4).

According to the scheme and calculated differences between the $\Delta^{13}C$ values of the three ecosystems, C from microaggregate $oDF_{1.6-2.0}$ goes to microaggregate $MF_{>2.0}$. Also, C from macroaggregates $MF_{>2.0}$ flows to $MF_{>2.0}$ of microaggregates. The first pathway can reflect the C

transformation in free microaggregates, whereas the second one is more probable for the microaggregates, which are formed inside the macroaggregates.

4. Discussion

4.1. Carbon distribution in aggregates and SOM density fractions

In this study we tested two hypotheses focused on the link between SOM formation and $\delta^{13}C$ values of C pools: 1) There is a continuous ^{13}C enrichment of OM during stabilization in soil because microbial utilization leads to preferential release of ^{12}C as CO_2 (Hobbie and Werner, 2004; Werth and Kuzyakov, 2010) and, consequently, the remaining C is enriched by ^{13}C. This hypothesis was supported based on increased $\delta^{13}C$ values of C in aggregates (Fig. 2 top) and increased $\Delta^{13}C$ of SOM fractions with increasing density (Fig. 3). The first hypothesis assumes the increase of $\delta^{13}C$ in both: i) total SOM by formation from litter, as well as ii) SOM pools by their formation within the individual steps. Consequently, the ^{13}C enrichment ($=\delta^{13}C$ increase) of one SOM fraction compared to the other shows that the first is the product of the second. Based on these ^{13}C enrichments between the fractions it is possible to follow the steps of SOM formation. Therefore, 2) our second hypothesis is that ^{13}C fractionation by individual steps of formation of SOM pools and fractions is relatively constant and is independent of ecosystem type. So, the ^{13}C fractionation *per se* (not the $\delta^{13}C$ of the fractions) depends only on the steps of C transformations and not on the C precursors. Consequently, minimal differences between $\Delta^{13}C$ of SOM fractions under various ecosystems correspond to maximal probability of the SOM formation pathways. We can test the consequences of the second hypothesis based on changes of $\Delta^{13}C$ of fractions separated by aggregate size and by density (Fig. 4).
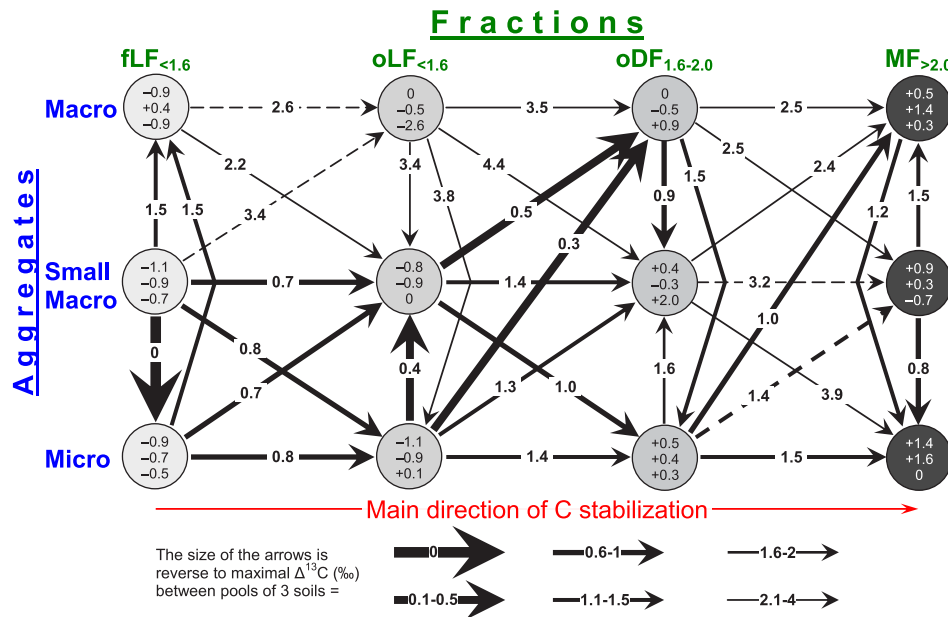

Fig. 4. Principal scheme of C flows in the system of aggregates and density fractions. The three values in circles show the difference between the $\delta^{13}C$ of the respective SOM fractions and the $\delta^{13}C$ of total SOM ($\Delta^{13}C$: the positive values reflect ^{13}C enrichment and negative values ^{13}C depletion of the fraction compared to the $\delta^{13}C$ of bulk SOM) and present the $\Delta^{13}C$ for soils of three ecosystems (from top to down): coniferous, deciduous forests, and arable land. The arrows show the directions of C flow based on the average increase of the $\Delta^{13}C$ values of the three ecosystems. Arrow size corresponds to the relative probability of C flow, whereas the numbers on the arrows show the exact calculated relative probability of the C flows for investigated soils. The smaller the $\Delta^{13}C$ differences between the three ecosystems (the minimal differences in $\Delta^{13}C$ between source and product for the ecosystems), the higher the probability of the ongoing process. (Only the 4 dashed lines are not in the accordance with the hypothesis that flows of C by stabilization go in the direction of $\Delta^{13}C$ increase. The other 29 lines correspond to the hypothesis.)

4.2. $\delta^{13}C$ values in aggregates and in density fractions

There was a trend (not significant) in decreasing $\delta^{13}C$ values from macro to microaggregates (Fig. 2 top). A similar result was observed in forest soils (John et al., 2005). Also, the ^{13}C NMR of aggregates in soils formed under coniferous forest, maize and grassland shows that various size classes do not differ in the degree of SOM decomposition (Helfrich et al., 2006). The fact that C in various aggregate size classes has a similar level of microbial processing contradicts existing theory that microaggregates contain more microbially transformed C than macroaggregates (Six et al., 2002). Three explanations can be forwarded for this result: 1) fast formation and breakdown of new aggregates compared to microbial processing of litter, 2) methodological problems − contamination of small aggregates with abrasive fine litter particles by dry sieving and slight dependence of aggregate composition (especially microaggregates) on the fractionation procedure (Denef et al., 2007) and 3) the continuous tillage of arable land leads to intensive mixing and disruption of aggregates.

Fast turnover of macroaggregates itself (around 5−33 days) was reported in experiments with Dysprosium-labeled tracer spheres and simultaneous application of fresh plant residues (Plante and McGill, 2002). Those authors noted that aggregate turnover was unaffected by the presence of fresh plant residues. This confirms the presence of SOM with similar decomposition stage in macro and microaggregates.

The second explanation involves methodological problems in dry vs. wet sieving, leading to redistribution of free POM between all aggregate size classes. Thus, low $\delta^{13}C$ values of free POM (Fig. 2 bottom) may contribute to low $\delta^{13}C$ of microaggregates (Fig. 2 top), yielding no significant $\delta^{13}C$ differences between micro- and macroaggregates. Furthermore, in our soils the C/N ratios in microaggregates were similar to or even higher than those in macroaggregates (Table 2). This additionally demonstrates the redistribution of undecomposed plant material ($fLF_{<1.6}$) between the aggregate size classes during dry sieving. The high C/N ratios in microaggregates can also be explained by the presence of two microaggregate types after dry sieving: 1) old one, containing microbially processed C and released from the crushed macroaggregates and 2) newly formed, containing less microbially processed C and released from the surface of macroaggregates (Ashman et al., 2003). Thus, the similar SOM composition of differently-sized aggregates isolated by dry sieving cannot alone describe the processes of C flows. Additional techniques, such as fractionation by density, are required to track the SOM transformations.

Because of DOC absence by the used dry sieving, we had no contamination of microaggregates with soluble C. Furthermore, there were no redistribution of POM from unstable macroaggregates between the other aggregate size classes. Both advantages of dry sieving were important to avoid contamination of isotopic composition of density fractions which were separated from the aggregate size classes.

The $\delta^{13}C$ values of the density fractions increased in the order: $fLF_{<1.6} < oLF_{<1.6} < oDF_{1.6−2.0} < MF_{>2.0}$ (Fig. 2 bottom) except in the arable soil. These results correspond to the continuous ^{13}C enrichment of the density fractions observed in many previous studies (Sollins et al., 2006; Crow et al., 2007; Dorodnikov et al., 2011). Such ^{13}C enrichment is the result of isotopic fractionation by progressive degradation of plant C with preferable decomposition of light ^{12}C to CO_2, whereas the microbial products remain enriched in ^{13}C (Werth and Kuzyakov, 2010; Blagodatskaya et al., 2011).

$\delta^{13}C$ values of $fLF_{<1.6}$ were the closest to the $\delta^{13}C$ of initial plant litter (Fig. 2 bottom) for the deciduous forest and arable soils, whereas it was not the case for the coniferous forest soil. The latter reflects a different localization of fresh litter inputs in coniferous forest vs. the two other soils. The presence of a thick litter O horizon in coniferous forest leads to the differentiation in the biochemical composition of top and bottom litter (Helfrich et al., 2006). Therefore, different $\delta^{13}C$ of fresh coniferous litter and already processed litter contributed to $fLF_{<1.6}$ (Fig. 2 bottom). Furthermore, leaching of soluble organic materials from O horizon into mineral horizons increases the $\delta^{13}C$ of stabilized C. This is because leached organic materials typically consist of carbohydrates, amino acids and carboxylic acids that are ^{13}C enriched compared to other compounds such as lignin or lipids (Hobbie and Werner, 2004).

Although $\delta^{13}C$ values of SOM fractions increased with density (Fig. 2 bottom), it is difficult to conclude which SOM fractions are sources and which are the products. Therefore, we calculated the $\delta^{13}C$ differences between SOM fractions and bulk soil ($\Delta^{13}C$) (Fig. 3). This showed that $fLF_{<1.6}$ and $oLF_{<1.6}$ had a similar $\Delta^{13}C$. This indicates a similar decomposition degree of C in these two fractions and the absence of strong biochemical changes during the formation of $oLF_{<1.6}$ from $fLF_{<1.6}$. The close chemical composition of $oLF_{<1.6}$ to original plant residues was shown by Kay (1990). Moreover, young plant C is preferentially accumulated in macroaggregates (Angers and Giroux, 1996), and this C was classified as an agent that temporarily binds macroaggregates (Tisdall and Oades, 1982). Assuming that macroaggregate turnover is fast (5−33 days, (Plante and McGill, 2002)), we expect rapid exchange of C between $fLF_{<1.6}$ and $oLF_{<1.6}$. Thus, the strong link of these two fractions with macroaggregate dynamics explains the similarity in the decomposition degree of C.

The C in $oDF_{1.6−2.0}$ had a $\Delta^{13}C$ enrichment similar to that in $MF_{>2.0}$ (Fig. 3). The results of the ^{13}C NMR for the density fractions under spruce, grassland and maize have shown an identical or lower alkyl-C/O-alkyl-C ratio in $oDF_{1.6−2.0}$ and $MF_{>2.0}$ compared to $fLF_{<1.6}$ (Helfrich et al., 2006). Such similarities are attributed to a similar degree of decomposition of these fractions (Baisden and Amundson, 2002).

With increasing particle density from 1.2 g cm^{-3} to >2 g cm^{-3}, the SOM fractions become more enriched by an average of 2‰. Assuming that C in $fLF_{<1.6} − oLF_{<1.6}$ and $oDF_{1.6−2.0} − MF_{>2.0}$ has a similar degree of decomposition, and that the most essential $\Delta^{13}C$ changes occur between $oLF_{<1.6}$ and $oDF_{1.6−2.0}$, then the biochemical transformation of soil C can be presented in the sequence $fLF_{<1.6} = oLF_{<1.6} \rightarrow oDF_{1.6−2.0} = MF_{>2.0}$.

We calculated the $\delta^{13}C$ differences between SOM fractions and bulk soil ($\Delta^{13}C$) for 70-values from other studies and related them to the particle density (Fig. 5). Over the broad range of soil types, ages and plant communities, the $\Delta^{13}C$ of SOM fractions increased with increasing density by 1.5−2‰. The main enrichment was in the density range 1.7−2 g cm^{-3}, whereas for the fractions with a density <1.7 g cm^{-3} and >2 g cm^{-3}, $\Delta^{13}C$ enrichment was less pronounced. Consequently, the highest degree of microbial transformations is ongoing for fractions with density between 1.7 and 2 g cm^{-3}. The less expressed $\Delta^{13}C$ enrichment for SOM fractions with density <1.7 g cm^{-3} is explained by a close link of these fractions with the aggregates' formation and disruption. Thus, these fractions can have a similar degree of microbial transformation. In contrast, for the fractions with a density >2 g cm^{-3}, other process can occur, namely the stable sorption of organic compounds on the mineral surfaces, preventing further microbial degradation of organic materials, or sorption of already ^{13}C enriched organic materials (Sollins et al., 2006).

The general trend of $\Delta^{13}C$ increase with increasing density of SOM fractions corresponds to the concept of soil C stabilization by association with mineral particles. Nonetheless, only the combination of $\Delta^{13}C$ in density fractions with aggregate hierarchy provided insights into C transformation and its pathways.
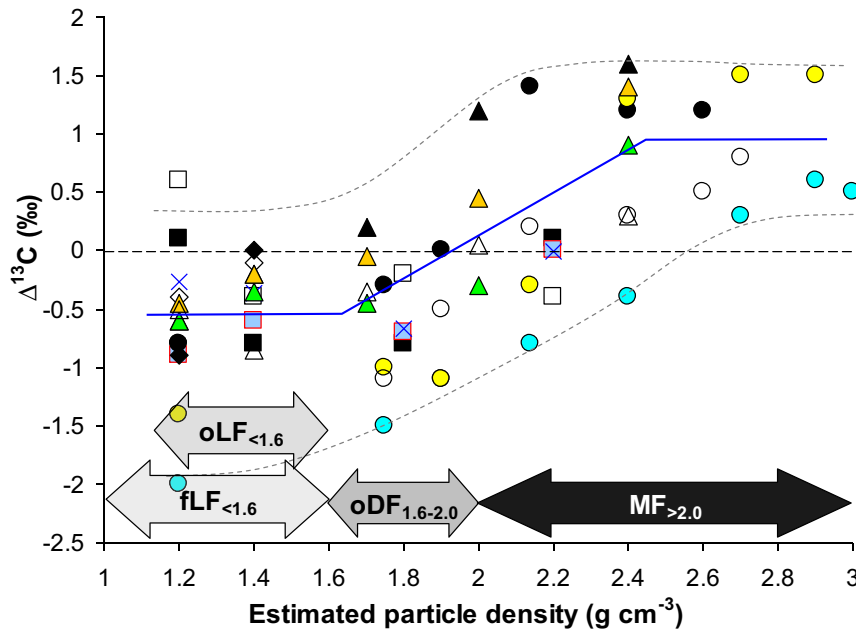
Fig. 5. $\Delta^{13}C$ values of SOM fractions (relative to $\delta^{13}C$ of bulk SOM) vs. particle density for soils from literature. The dotted lines are held through the minimum and maximum $\Delta^{13}C$ values, whereas the solid line through the mean $\Delta^{13}C$ values.

4.3. Principal concept of C flows within aggregates and SOM fractions

To test our second hypothesis we created scheme (Fig. 4) based on 1) the existing concepts of aggregate hierarchy and 2) the information about the "sources" and "products" of organic materials in SOM fractions according to hypothesis one.

The proposed concept assumes that the closer the differences of ^{13}C fractionation between the respective SOM pools in three ecosystems, the higher the probability of ongoing C flow. Generally, $\Delta^{13}C$ values of the 12 aggregates and SOM density fractions increased from the top-left to the right bottom (Fig. 4). $fLF_{<1.6}$ (lowest $\Delta^{13}C$) was a starting point of C flows in the aggregate system, and $MF_{>2.0}$ (highest $\Delta^{13}C$) was the end. In general, these C flows agree with the most models of aggregate formation based on the plant litter C (Oades, 1984; Golchin et al., 1998; Six et al., 2002). Our approach, however, allows to show the complexity of the processes of C transformation: multidirectional pathways of C between and within density fractions of differently sized aggregates are possible.

The initial C flow starts from $fLF_{<1.6}$, which represents the slightly decomposed plant litter (Golchin et al., 1998). The C in $fLF_{<1.6}$ flows from the small macroaggregates to microaggregates and also between both macroaggregate classes (Fig. 4). These flows

are in accordance with observations that fresh plant residues are the main agent for macroaggregate formation (Six et al., 2002). Also, the litter distribution between aggregate size classes has shown that plant residues are first associated with macroaggregates and are then redistributed to microaggregates (Angers et al., 1997).

One previous assumption was that $oLF_{<1.6}$ is more microbially processed than $oDF_{1.6-2.0}$ (Golchin et al., 1998) because $oLF_{<1.6}$ originated by weakening of interactions between minerals and organic substances due to decomposition of $oDF_{1.6-2.0}$. Our results contradict the model of Golchin et al. (1998) because we observed strong ^{13}C enrichment in $oDF_{1.6-2.0}$ vs. $oLF_{<1.6}$ (Fig. 2 bottom and 3). This clearly reflects biochemical changes between these fractions and agrees with the reported $\delta^{13}C$ increase of SOM fractions as their density increases (Baisden and Amundson, 2002). Baisden and Amundson (2002) however, showed the same ^{14}C age of $oLF_{<1.6}$ and $oDF_{1.6-2.0}$. They therefore proposed that occluded POM fractions with various densities exist in soil simultaneously.

The C flows from $oDF_{1.6-2.0}$ to $MF_{>2.0}$ and also within $MF_{>2.0}$ can be explained by the aggregate formation theory of Six et al. (2000). The C flows from microaggregate $oDF_{1.6-2.0}$ to macroaggregates $MF_{>2.0}$ are assumed for the microaggregates, which are formed inside the macroaggregates. At the same time, C flow from microaggregate $oDF_{1.6-2.0}$ to microaggregate $MF_{>2.0}$ confirms the C

transformations in free microaggregates (Six et al., 1998; Ashman et al., 2003). The C flows within $MF_{>2.0}$ are mainly directed from macro to microaggregates. This also agrees with C flows for microaggregates, which are formed inside the macroaggregates (Jastrow, 1996; Six et al., 1998).

Concluding, the $\Delta^{13}C$ values of the SOM fractions as well as the ^{13}C fractionation between the fractions clearly show that C transformations are ongoing from macro to microaggregates and furthermore to $oDF_{1.6-2.0}$ and $MF_{>2.0}$. These concordant results were obtained even though SOM formation was compared in three completely different ecosystems: coniferous and deciduous forests as well as arable land. The C/N ratios of SOM fractions partly confirm these C flows (Fig. 1 bottom).

4.4. Advantages and limitations of the ^{13}C natural abundance approach for analysis of steps of C transformation in soil

The proposed concept of C flows during SOM formation was developed using solely the natural changes of the $^{13}C/^{12}C$ ratios, from which the probable C pathways between the aggregates and SOM fractions were suggested. In contrast to the application of ^{13}C- or ^{14}C-labeled plant residues and following their incorporation in distinct pools and respective turnover, our study is based on long-term C input and transformation in soil under natural conditions. In the former, the applied ^{13}C- or ^{14}C-labeled litter represents the pulse input of tracers. In contrast, the $\delta^{13}C$ approach suggested here is based on subsequent ^{13}C fractionation during formation of SOM pools.

Our study was conducted with soils of the boreal zone. The proposed pathways can be different for soils with other mechanisms of aggregate formation, e.g. abiotic ones such as drying–rewetting cycles, physico-chemical bounding on Fe, Al, or Ca, etc. Because different C stabilization mechanisms dominate under arid/semiarid climatic conditions compared to soils under temperate/boreal climates, the ^{13}C fractionation is different in C_3 and C_4 soils (Werth and Kuzyakov, 2010). This calls for further confirmation of the $\delta^{13}C$ approach suggested here.

5. Conclusions

We suggested and demonstrated the validity of a new approach for tracing C flows between SOM fractions based on natural differences in stable C isotopic composition. According to ^{13}C enrichment between the fractions, we predicted the steps of SOM transformation. The $\delta^{13}C$ values of $fLF_{<1.6}$ and $oLF_{<1.6}$ POM were close to those of the $\delta^{13}C$ of the initial plant material. Similar $\Delta^{13}C$ values of these two fraction point to their similar chemical composition and to the absence of strong biochemical transformations during their formation. Two heavy fractions – $oDF_{1.6-2.0}$ and $MF_{>2.0}$ – were more $\Delta^{13}C$ enriched compared with both light fractions. This means that these heavy fractions were microbial transformation products of the lighter fractions ($fLF_{<1.6}$ and $oLF_{<1.6}$).

We hypothesized that ^{13}C fractionation during individual steps of formation of SOM pools and fractions are relatively constant, independent on the ecosystem type. To elucidate this, we placed the SOM fractions in a conceptual scheme that allowed us to quantify C flows within the aggregate/SOM fraction system. We revealed that the main flows of C in the SOM fractions go from $fLF_{<1.6}$ to $MF_{>2.0}$ through $oLF_{<1.6}$ and $oDF_{1.6-2.0}$. The most intensive C flows occur in the small macro and microaggregate size classes. C transformation in $oDF_{1.6-2.0}$ and $MF_{>2.0}$ proceeds preferentially in microaggregate size classes. In conclusion, the suggested $\delta^{13}C$ natural abundance approach based on ^{13}C fractionation within individual steps of SOM formation is very useful and probably the

sole approach to estimate C flows under steady state without ^{13}C or ^{14}C labeling.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.soilbio.2014.01.011.

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