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¹³C isotopic signature and C concentration of soil density fractions illustrate reduced C allocation to subalpine grassland soil under high atmospheric N deposition

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Abstract

We followed soil C fluxes in a subalpine grassland system exposed to experimentally increased atmospheric N deposition for 7 years. Earlier we found that, different from the plant productivity response, the bulk soil C stock increase was highest at the medium, not the high N input as hypothesized. This implies that a smaller N-deposition rate has a greater potential to favor the biological greenhouse gas-sink. To help elucidate the mechanisms controlling those changes in SOC in response to N deposition, we produced four soil density fractions and analyzed soil organic C concentration [SOC], as well as $\delta^{13}\text{C}_{\text{SOC}}$ of SOC components. Soil respired CO_2 ($\delta^{13}\text{C}_{\text{CO}_2}$) was analyzed to better distinguish seasonal short term dynamics from N-deposition effects and to identify the predominant substrate of soil respiration.

Both at the start of the experiment and after 7 years we found a strong, negative correlation between [SOC] and $\delta^{13}\text{C}_{\text{SOC}}$ of the soil density fractions in the control treatment, consistent with an advanced stage of microbial processing of SOC in fractions of higher density. During the experiment the [SOC] increased in the two lighter density fractions, but decreased in the two heavier fractions, suggesting a possible priming effect that accelerated decomposition of formerly recalcitrant (heavy) organic matter pools. The seasonal pattern of soil $\delta^{13}\text{C}_{\text{CO}_2}$ was affected by weather and canopy development, and $\delta^{13}\text{C}_{\text{CO}_2}$ values for the different N treatment levels indicated that soil respiration originated primarily from the lightest density fractions.

Surprisingly, [SOC] increases were significantly higher under medium N deposition in the <1.8 fraction and in bulk soil, compared to the high N treatment. Analogously, the depletion of $\delta^{13}\text{C}_{\text{SOC}}$ was significantly higher in the medium compared to the high N treatment in the three lighter fractions. Thus, medium N deposition favored the highest C sequestration potential, compared to the low N control and the high N treatment.

Clearly, our results show that it is inappropriate to use plant productivity N response as an indicator for shifts in SOC content in grassland ecosystems. Here, isotopic techniques illustrated why atmospheric N deposition of $14 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ is below, and $54 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ is above a threshold that tips the balance between new, assimilative gains and respiratory losses towards a net loss of [SOC] for certain soil fractions in the subalpine grassland.

Keywords

Stable C isotopes; density fractions; soil respiration; CO_2 ; seasonal dynamics; Nitrogen deposition; C sequestration

1. Introduction

Grassland soils can act as either a biological carbon (C) sink or a source, and the balance between soil carbon sequestration and emission feeds back on atmospheric CO_2 concentration. Globally soil respiration alone emits c. ten times more CO_2 to the atmosphere than fossil fuel combustion (Schlesinger and Andrews, 2000). Altered C assimilation and ecosystem respiration rates can substantially alter the size of the C stock in soils, thus affecting the extent to which soils serve as a C sink. Atmospheric N deposition (e.g. from air pollution) can increase plant productivity and thus C acquisition potential, but it may also increase respiratory C losses from autotrophic and heterotrophic organisms, counteracting any net increase in C storage. Understanding the response of C fluxes to and from soils under increased atmospheric N deposition, and hence whether they serve as C sinks or sources, is therefore of high importance.

To achieve a differentiation of the bulk soil organic carbon (SOC) response to environmental change, a density fractionation of the bulk soil can be applied. This generates distinct classes of SOC pools. Light fractions ($<1.6\text{--}2 \text{ g cm}^{-3}$) consist mostly of particulate organic matter (POM) and are considered to have short turnover times, i.e. they are decomposed more easily, while heavier fractions ($>1.6\text{--}2 \text{ g cm}^{-3}$) contain mineral associated soil organic matter with longer turnover times (Christensen, 1992; von Lützow et al., 2007). Density fractions were also shown to differ in [SOC], age and isotopic signature (Baisden et al., 2002; Sollins et al., 2009). The soil fractions commonly have lower [SOC], enriched (i.e., greater) $\delta^{13}\text{C}$ signatures and a higher age with increasing density.

Plant C entering the soil has a very heterogeneous $\delta^{13}\text{C}$ signature, that reflects discrimination against ^{13}C in stomatal uptake, carboxylation, and post photosynthetic fractionation (Farquhar et al., 1982; Farquhar et al., 1989; Badeck et al., 2005). $\delta^{13}\text{C}$ of assimilates is less negative under hot and dry summer conditions or highly productive situations with a low internal leaf CO_2 concentration, compared to conditions with a high CO_2 concentration. As a result, plant C inputs to soil are significantly more depleted in ^{13}C (more negative $\delta^{13}\text{C}$) than the $\delta^{13}\text{C}$ signature of atmospheric CO_2 .

When the organic C concentration in the soil decreases over time through decomposition, the $\delta^{13}\text{C}$ of the remaining SOC increases again (i.e., becomes less negative; Menichetti et al., 2015). This is commonly observed along depth gradients in soils (Wynn et al., 2006) and

is related to the preferential substrate use of ^{12}C and kinetic fractionation of the heavier ^{13}C isotope in decomposition processes. Ultimately, the increase in $\delta^{13}\text{C}$ can serve as a measure for the number of steps that the organic material has moved through in the microbial foodweb (Yang et al., 2014).

Finally, soil respired CO_2 from both autotrophic (i.e., root) respiration and heterotrophic (i.e., microbial and fungal) respiration carries a $\delta^{13}\text{C}$ isotopic signature that is very similar to the substrate utilized (Šantrůková et al., 2000). Therefore, also the $\delta^{13}\text{C}$ of soil respired CO_2 reflects the climatic conditions at the time when the C substrate was originally assimilated and the decomposition history of the substrate. This was demonstrated for contrasting climate zones and also as a seasonal effect (e.g. Bowling et al., 2002; Pataki et al., 2003).

Alpine grasslands tend to be N limited, so growth would be expected to respond to increasing N availability, affecting in turn the amount and isotopic signature of C reaching the soil. Thus, characterizing C concentration changes and the isotopic composition of soil density fractions, along with seasonal changes in temperature, moisture and other factors, can provide valuable mechanistic information on critical soil C flux processes, affected by atmospheric N deposition.

In an earlier analysis of the Alp Flix field experiment in a species rich, high-elevation grassland ecosystem, we found that after seven years, likely as an effect of management change from grazing to cutting, bulk soil (0–20 cm depth) C stock increased from 6.9 to 7.5 kg m^{-2} in the $\text{N4}_{\text{control}}$ treatment ($4\text{kg N ha}^{-1} \text{yr}^{-1}$ background deposition). The atmospheric N deposition treatment led to a significant, monotonous increase of harvested plant C with increasing N deposition rate (Volk et al., 2016). But the concomitant increases in bulk SOC concentration and stock were not significant and the largest increase was found in the medium N14 deposition treatment ($14\text{kg N ha}^{-1} \text{yr}^{-1}$), not at the high N54-deposition ($54\text{kg N ha}^{-1} \text{yr}^{-1}$). Because absolute plant C input from dry matter above and below ground was always largest in the high N54 treatment, the smaller soil C stock was assumed to result from increased respiration (Volk et al., 2016).

This new study utilizes the $\delta^{13}\text{C}$ signatures and the C concentration of distinct, density separated soil fractions, as well as $\delta^{13}\text{C}$ signatures of soil respired CO_2 to better explain the processes controlling soil carbon fluxes under atmospheric N deposition and aims at providing a consistent interpretation of $\delta^{13}\text{C}$ values across seasons and N treatments.

Based on the findings cited above we hypothesized that:

1. The bulk soil [SOC] increase in the $\text{N4}_{\text{control}}$ treatment includes increases in all density fractions and is associated with lower $\delta^{13}\text{C}$ values. We expect the strongest effects on [SOC] and $\delta^{13}\text{C}_{\text{SOC}}$ in the lightest fractions, identifying them as those SOC pools that contain the most newly imported C.
2. The plant growth response to the N deposition treatment creates a proportionally large input of newly assimilated C with a very negative $\delta^{13}\text{C}$ value, thus causing the strongest [SOC] increase and the most negative isotopic signature in the N54 treatment.

3. The $\delta^{13}\text{C}$ signature of soil respired CO_2 reflects the effect of seasonally changing growing conditions on ^{13}C discrimination and matches the $\delta^{13}\text{C}_{\text{SOC}}$ of the lightest, presumably biologically most active, density fraction.

2. Methods

2.1. Experimental site and climatic conditions

Our study was conducted on a grassland plateau (46°31'51.3"N 9°39'07.2"E; Alp Flix, Sur, Canton Grisons, Switzerland) at 1900 m above sea level (a. s. l.) in the Central Alps. The local climatic tree line is at ~2200 m a. s. l. For at least 60 years prior to the experiment, the low-intensity management included 3–4 weeks of cattle grazing at ~1.3 livestock units ha^{-1} . No manure or fertilizer was applied. Snow cover lasts from December until April. Annual mean temperature during the seven-year experiment was 1.1 °C, with an April to October mean of 6.2 °C. Mean April to October precipitation was 853 mm. Background N deposition (air, rainwater and snow) amounts to <4 $\text{kg N ha}^{-1} \text{yr}^{-1}$ (Bassin et al., 2007). Further information on the physical conditions in the environment can be found in Volk et al. (2014).

2.2. Plots and treatments

180 turf monoliths ($L \times W \times H = 30 \times 40 \times 22 \text{ cm}$) were carefully excavated in October 2003, placed in drained plastic boxes and randomly assigned to treatment combinations. Groups of 20 were placed in nine pits, to fit flush with the surface. To minimize confounding effects of microclimatic differences between pits, monoliths were re-randomized annually between pits. N deposition loads were 4 (control), 9, 14, 29, and 54 $\text{kg N ha}^{-1} \text{yr}^{-1}$, including the 4 $\text{kg N ha}^{-1} \text{yr}^{-1}$ background deposition at the site. For the purpose of discussion, the treatments are termed $\text{N4}_{\text{control}}$, N9, N14, N29, and N54, respectively. From 2004 to 2010 N additions were applied bi-weekly during the snow-free period, from May to October, by adding 200 ml ammonium nitrate (NH_4NO_3)-amended well water to each monolith. $\text{N4}_{\text{control}}$ monoliths received only water. In this study we evaluated only N treatment levels $\text{N4}_{\text{control}}$, N14 and N54, represented by 108 out of the 180 monoliths.

2.3. Plant and soil sampling

Aboveground plant biomass was cut annually during peak canopy development (end of July), 2 cm above the ground. The harvested material was oven dried at 60°C and weighed. Bulk leaf material C concentration and $\delta^{13}\text{C}$ values were calculated from cover weighted values of individual species collected in 2006 (Bassin et al., 2009). Roots for C analysis were collected in 2010, following the sieving of bulk soil.

In October 2003 and in October 2010, soil from 0–10 cm depth was sampled. All samples were dried and sieved (2 mm). We lumped soil samples from four monoliths, receiving each the same N treatment, so that the total of 108 monoliths resulted in 27 samples and yielded $n = 9$ per each of the three N treatment levels.

2.4. Soil density fractionation

Sieved soil (2mm) from the 0–10 cm depth layer was subjected to density fractionation (Sohi et al., 2005) with sodium polytungstate (SPT, $\text{Na}_6[\text{H}_2\text{W}_{12}\text{O}_{40}]$) solution, yielding a

total of four fractions (US EPA, 2003, SOP-WED/PCEB/BB/0601-000; Swanston et al., 2005). Bulk soil also was investigated. SPT solutions were used in this sequence of densities: 2.2 g cm^{-3} , 1.4 g cm^{-3} and 1.8 g cm^{-3} . Soil was sequentially dispersed in SPT/demineralized water solution with an ultrasonic converter set to 22J/ml. The dispersion was centrifuged and the resulting heavy or light fractions were either re-suspended in the next SPT solution or represented one of the final soil density fractions. Using this approach, the following soil density fractions were produced: $<1.4 \text{ g cm}^{-3}$, $1.4\text{--}1.8 \text{ g cm}^{-3}$, $1.8\text{--}2.2 \text{ g cm}^{-3}$, $>2.2 \text{ g cm}^{-3}$. These fractions will be referred to as: <1.4 , <1.8 , >1.8 and $>2.2 \text{ g cm}^{-3}$ fractions.

2.5. Soil air sampling

In 81 of the monoliths (i.e. 27 per N treatment), we buried low-volume, fritted Swagelok gas wells at a depth of 8 cm in the soil to sample soil pore air. The gas wells had a 1 mm inner diameter tube with a Luer Lock cone protruding from the soil, allowing us to take gas samples without disturbing the soil. CO_2 -samples were regularly withdrawn from the cone for isotopic analyses by using a 3-way valve syringe and subsequently injected into a septum-capped 12 ml Exetainer glass vial (He flushed, with exit needle). The volume drawn from the gas wells was generally 15 ml per sample to avoid incursions of aboveground air. Gas samples were collected on six days during the 2010 snow-free period. Given the location of our gas wells in the soil, the C-isotopic composition of the gases sampled actually represented soil pore air, which was enriched in ^{13}C due to the faster diffusion of $^{12}\text{CO}_2$ from the soil at the surface. In order to estimate the isotopic composition of soil respiration, we calculated the values by subtracting 4.4 per mil to correct for the faster diffusion of the lighter isotope (Cerling, et al., 1991). For the purpose of discussion, diffusion-corrected $\delta^{13}\text{C}_{\text{CO}_2}$ values will be considered an estimate of soil respired CO_2 .

2.6. SOC concentration and C isotopic ratio analyses

Plant material and soil organic matter was analyzed for elemental C/N and $^{13}\text{C}/^{12}\text{C}$ ratios using an elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer (EA-IRMS) (Thermo Fisher Scientific, Bremen, Germany). Prior to the C isotope analysis, homogenized, dry soil samples were acidified with dilute HCl (1 N), washed with deionized water to remove inorganic carbonates, and dried in the oven at 50°C . Sample material was combusted in the presence of O_2 at 1030°C . Combustion gases were purified and transferred to the IRMS. Soil gas CO_2 was sampled into 12 ml Exetainers (Labco Limited, Lampeter, UK) that were purged with helium and were evacuated. The C isotope composition was determined on-line using a Gasbench II (Thermo Fisher Scientific, Bremen, Germany) coupled to an IRMS. Organic C isotope measurements were calibrated using the international standards NBS19 and NBS22. C isotope ratios of soil CO_2 were calibrated using international carbonate standards NBS19 and LEVEC, which were reacted with anhydrous phosphoric acid (103%) at 72°C for 90 minutes. All stable carbon isotope ratios are reported in the conventional $\delta^{13}\text{C}$ -notation relative to the Vienna Pee Dee Belemnite (VPDB) carbonate standard. Since all values discussed in biological contexts are ^{13}C depleted compared to the VPDB standard, the terminology used here focusses on the degree of depletion in the $^{13}\text{C}/^{12}\text{C}$ isotope ratio. Based on replicate analyses of samples and carbon

isotope standards, the analytical reproducibility for $\delta^{13}\text{C}$ organic and $\delta^{13}\text{C}_{\text{CO}_2}$ was 0.1‰ and 0.2‰, respectively.

2.7. Statistical analyses

SOC concentrations and $\delta^{13}\text{C}$ variations in density fractions and bulk soil between 2003 and 2010 were tested for probability of equal means using a two-sided, paired Student's t-test using control treatment sample data. The N deposition effect was tested using a one way ANOVA on the difference of values in 2003 and 2010 for the individual plots. Data were Box-Cox-transformed when necessary or alternatively a non-parametric ANOVA on ranks (Kruskal-Wallis) was applied. When equal means or medians were rejected in omnibus ANOVA, planned comparisons were used to test against equal means between individual N treatment levels.

Seasonal differences and the N-treatment effect on respired CO_2 $\delta^{13}\text{C}$ were first tested in a repeated measures ANOVA (RM). N treatment and sampling campaign date were integrated as a categorical variable. Technical problems with gas wells made it necessary to exclude some monoliths from the RM that were not available for every sampling day. Differences between means of N treatment levels at individual sampling campaign dates were tested using planned comparisons following one-way ANOVA. Analyses were carried out using the NCSS software package (NCSS 7.1, USA, 2007). Values in the text and the figures are group means ± 1 standard error (SE).

3. Results

3.1. SOC concentration and $\delta^{13}\text{C}_{\text{SOC}}$ of soil density fractions

3.1.1. Control treatment—In both sampling years (2003 and 2010) we observed higher [SOC] and more depleted $\delta^{13}\text{C}$ signatures ($\delta^{13}\text{C}_{\text{SOC}}$) with decreasing density of the SOC fractions. This resulted in a strong, linear regression of [SOC] and $\delta^{13}\text{C}_{\text{SOC}}$ of the respective fractions (Fig. 1 A, B). Also the non-fractionated bulk soil samples, representing the weighted means of all fractions, perfectly fit this regression. The $\delta^{13}\text{C}$ of bulk roots ($\delta^{13}\text{C}_{\text{RT}}$), analyzed in 2010, was $-26.3\text{‰} \pm 0.11$, ranging in the middle between density fractions $\delta^{13}\text{C}_{\text{SOC}}$.

We also found a significant change in the [SOC] between the initial sampling and the second sampling seven years later. [SOC] significantly increased in the two light soil density fractions (<1.4 , <1.8) and in bulk soil (Table 1 and Figure 2 A), suggesting net carbon sequestration in the studied soils. Surprisingly, the C concentration significantly decreased in the heavier fractions (>1.8 , >2.2). But regardless of a gain or loss of soil C in the single fractions, $\delta^{13}\text{C}_{\text{SOC}}$ of all density fractions and of the bulk soil was significantly lower after seven years (Table 1).

3.1.2. N-deposition treatment—A significant positive effect of N deposition on [SOC] was observed in the <1.8 fraction ($P=0.033$) and in the bulk soil ($P=0.032$; Fig. 2 A). Interestingly, the strongest stimulation was observed in the N14, not in the N54 treatment. C concentrations also increased significantly ($P=0.015$) in the roots from 38.7% (± 0.23 , N4) to 39.2% (± 0.24 , N14) and to 40.0% (± 0.34 , N54). Also under increased N deposition

[SOC] dropped in the two heavier soil fractions, like it did in the N4_{control} treatment. The N deposition treatment significantly ($P = 0.029$) reduced $\delta^{13}\text{C}_{\text{SOC}}$ in all soil density fractions and in bulk soil (Fig. 2 B). $\delta^{13}\text{C}_{\text{SOC}}$ in the N14 treatment was lower than in the N54 treatment, except for the >2.2 fraction and bulk soil. No N-effect was found for $\delta^{13}\text{C}_{\text{RT}}$ of bulk roots.

3.2. $\delta^{13}\text{C}$ of soil respired CO_2 ($\delta^{13}\text{C}_{\text{CO}_2}$)

Soil respired $\delta^{13}\text{C}_{\text{CO}_2}$ underwent significant seasonal changes, with the least depleted $\delta^{13}\text{C}_{\text{CO}_2}$ signatures at the end of the winter just after snowmelt and again under mature canopy conditions at harvest time in summer (Fig. 3). The strongest ^{13}C depletion was found early and late in the growing season. Repeated measures ANOVA for the whole season yielded a significant N-effect on $\delta^{13}\text{C}_{\text{CO}_2}$ (Table 2). Post hoc tests indicated significantly less depleted $\delta^{13}\text{C}_{\text{CO}_2}$ signatures in the N54 treatment for the first two measurement campaigns in spring and again for the last one late in the fall (Fig. 3).

4. Discussion

4.1. Microbial metabolism and soil density fractionation: N4_{control} treatment

The strong correlation between [SOC] and $\delta^{13}\text{C}_{\text{SOC}}$ in both years in the N4_{control} treatment suggests that $\delta^{13}\text{C}$ isotopic signature is a proxy for soil history with respect to the degree of C stabilization and turnover in microbial metabolism. Because the $\delta^{13}\text{C}_{\text{SOC}}$ signature of soil carbon pools responds to a multitude of physical and biological factors, gradients in $\delta^{13}\text{C}_{\text{SOC}}$ require careful interpretation. It took the biologically and environmentally insensitive radiocarbon dating technology, paralleled by $\delta^{13}\text{C}$ analyses (Balesdent and Mariotti, 1996; Ladyman and Harkness, 1980) to eventually establish an unambiguous relationship of increasing SOC age with increased enrichment of ^{13}C in bulk soil samples. Drawing on soil sample archives from five European long-term, 27–80 years bare fallow experiments, Menichetti et al. (2015) demonstrated how soil $\delta^{13}\text{C}_{\text{SOC}}$ increased when the C resources for microbial respiration became increasingly exhausted and [SOC] declined. Research based on radiocarbon analyses also found links between increasing density in fractionated soils and increased mean SOC residence times, higher $\delta^{13}\text{C}_{\text{SOC}}$ values, and higher degrees of organic matter decomposition (Baisden et al., 2002; Meyer and Leifeld, 2013; Sollins et al., 2009). Based on these studies, we are confident that increasingly dense fractions and enriched $\delta^{13}\text{C}_{\text{SOC}}$ signatures observed here constitute distinct SOC pools that reflect progressively later stages of decomposition and stronger mineral association. Likewise, a depleted $\delta^{13}\text{C}_{\text{SOC}}$ signature in these fractions indicates the presence of more labile and younger (i.e., recently assimilated) SOC.

After seven years and an associated $0.6 \text{ kg m}^{-2} \text{ C}$ gain in the grassland bulk soil (Volk et al., 2016) there was a significant increase in [SOC] in the two light fractions, which was accompanied by a parallel decrease in $\delta^{13}\text{C}_{\text{SOC}}$. But the two heavier fractions showed decreased [SOC] despite a more negative (decreased) $\delta^{13}\text{C}_{\text{SOC}}$ signature (compare Tab. 1). This is not consistent with the notion that $\delta^{13}\text{C}_{\text{SOC}}$ generally increases with decreasing soil C concentration and not in line with our first hypothesis. Nevertheless, the correlation between [SOC] and $\delta^{13}\text{C}_{\text{SOC}}$ remained intact across fractions (Fig. 1 A, B). We suggest that

the decline of C concentration is indicative of a stimulation of the microbial C mineralization (priming) process. This may be a metabolic priming (Fontaine et al., 2003; Kuzyakov et al., 2000), where the input of a small amount of new, readily available C leads to the decomposition of previously protected organic C. In addition, as Keiluweit et al. (2015) suggested, a priming effect can result from the release of (e.g.) oxalic acid from the roots. This reduces the strong association of organic compounds with minerals and thus increases the availability of a substrate with a relatively enriched $\delta^{13}\text{C}$ signature. The increased decomposition of such enriched substrates would then result in a negative C balance for the fraction and leave a more ^{13}C depleted isotope mixture behind, as observed in this study. Thus, in our grassland, metabolic priming is a possible mechanism for triggering the increased release of older and more protected SOC to the atmosphere. As a consequence, the import of 'new' C may have an antagonistic effect on the C storage of light vs. heavy density fractions: The lighter the fractions, the higher the import rate of newly assimilated C and the lower the proportion of recalcitrant organic C that is prone to priming effects, resulting in a positive C balance for the fraction. But the heavier the fraction, the smaller the C import and the higher the potential for mobilization of previously recalcitrant organic C, resulting in net C loss from that fraction.

4.2. Soil density fraction response to N deposition treatment

As an effect of N deposition, [SOC] gains were larger - in light fractions representing new C - and losses were smaller (statistically not significant) - in heavy fractions comprised of older C - in N14 compared to N54. Despite the Shoot/Root ratio shift towards lower belowground biomass with increasing N deposition, as found in the previous study, the absolute root mass was always highest in N54 (Volk et al., 2014). This means that the lower [SOC] is likely not a consequence of reduced C input, but of increased metabolic turnover rates at N54. An earlier ecosystem CO_2 exchange parameterization for the whole seven-year period provides evidence for this effect: We found that the N-effect ($N_{\text{treatment}}/N_{\text{control}}$) on gross primary productivity (GPP) differed only marginally between the N14 and the N54 treatment by a factor of 1.1. The N-effect on ecosystem respiration on the other hand increased by a factor of 1.8, showing a maintained response of respiration even at higher N deposition rates (Volk et al., 2016). For example, in a warm and dry growing season, when the ecosystem CO_2 balance was generally negative, N54 grassland plots lost 48 g C m^{-2} more than the N14 plots (Volk et al., 2011).

In the three lighter, biologically more active fractions (<1.4 , <1.8 and >1.8) with shorter turnover times, the relationship of higher soil C concentrations and lower $\delta^{13}\text{C}_{\text{SOC}}$ signatures matched expectations when compared between the N14 and N54 treatments. But in comparison with N_{control} the $\delta^{13}\text{C}_{\text{SOC}}$ under N54 was considerably less depleted in the lightest (<1.4), presumably biologically most active soil density fraction, despite very similar [SOC] values. This may have resulted from a change in species composition in favor of sedges, increasing the aboveground biomass of this functional group under N54 by a factor of 3.6 (Bassin et al., 2013). Leaves of the most abundant sedge *Carex sempervirens* were c. 1.4‰ more enriched in ^{13}C than the average leaf material and in the N54 treatment the $\delta^{13}\text{C}$ was another 0.6‰ higher compared to the control treatment (Bassin et al., 2009). This implies that the increased abundance of *C. sempervirens* could have strongly

contributed to the less than expected decrease of the $\delta^{13}\text{C}_{\text{SOC}}$ signature in the N54 treatment. In consequence, not the largest plant C input in the N54 treatment determined the associated $\delta^{13}\text{C}_{\text{SOC}}$, but the [SOC] of the respective treatment, resulting from the soil C balance. An exception to this rule was likely caused by an N driven shift in species composition.

4.3. $\delta^{13}\text{C}$ of soil respired CO_2

Some of the highest $\delta^{13}\text{C}_{\text{CO}_2}$ values were observed early in the year, prior to snowmelt, indicating that older C sources were being used as microbial respiratory substrate. Generally, it can be assumed that $\delta^{13}\text{C}_{\text{CO}_2}$ approximately reflects the $\delta^{13}\text{C}$ of the substrate predominantly metabolized (Šantrůková et al., 2000). In our experiment, prior to the growth period, just after snowmelt and before plant productivity increases, the high $\delta^{13}\text{C}_{\text{CO}_2}$ values in early spring may reflect availability of labile substrates from microbial cell lysis following winter freezing (Conen et al., 2008). These labile substrates would be available before the more ^{13}C depleted, new C, assimilated by the developing canopy, becomes available as root exudates. In addition, the highly ^{13}C depleted substrates generated during the last growing period may simply be exhausted by the end of the winter.

$\delta^{13}\text{C}_{\text{CO}_2}$ signatures started increasing again in mid-May until mid-August, before decreasing in mid-October (Fig. 3). The same pattern was reported for the $\delta^{13}\text{C}_{\text{CO}_2}$ of soil and ecosystem respiration across seasonal and geographic climate gradients by numerous authors (e.g. Bowling et al., 2002; Scartazza et al., 2004; Wingate et al., 2010). On a shorter timescale, an analogous effect was found in diel dynamics of $\delta^{13}\text{C}_{\text{CO}_2}$ (Kodama et al., 2008; Werner et al., 2006; Werner and Gessler, 2011).

Such seasonal dynamics of $\delta^{13}\text{C}_{\text{CO}_2}$ require that there is either a rapid change of the $\delta^{13}\text{C}$ of the metabolized substrates during the season (e.g., due to environmental/seasonal growth effects), or that the decomposer community chooses different substrates (preferential microbial consumption) during different periods of the year. There is good evidence for a change of the $\delta^{13}\text{C}$ of substrates, because under increasingly warm and dry weather conditions and/or during high plant growth rates, the photosynthetic ^{13}C discrimination is more controlled by stomatal and mesophyll diffusion (-4.4‰) and less controlled by the carboxylation reaction through RuBisCo (-30‰) (Farquhar et al., 1989). This environmental signature in photosynthetic products consistently translates into C3 plant organs (Murphy and Bowman, 2009). Equivalent to the gradient found in soil CO_2 in our experiment, also Wang and Schjoerring (2012) demonstrated an annual 2‰ gradient of $\delta^{13}\text{C}$ for ryegrass plant organs, with lowest levels occurring early in the season and greatest $\delta^{13}\text{C}$ values occurring during the summer. Jäggi et al. (2005) observed a similar enrichment (higher values) associated with lower soil moisture/stomatal conductance values in the $\delta^{13}\text{C}$ of grassland species leaves.

If the dynamics that we observed for $\delta^{13}\text{C}_{\text{CO}_2}$ are driven by the suggested environmental effects on photosynthetic discrimination, then the substrate needs to be available for respiration within days after synthesis. We assume that the $\delta^{13}\text{C}$ of solid above- and belowground litter, accumulated over many years and available in various stages of decomposition, integrates the whole range of seasonal effects on photosynthetic

fractionation. Therefore, solid litter is unlikely to reflect the transition from predominantly carboxylation driven fractionation towards diffusion driven fractionation over short time intervals and must thus be excluded from the substrates that are potentially responsible for seasonal soil $\delta^{13}\text{C}_{\text{CO}_2}$ dynamics. But in grasslands up to 40% of photosynthates are transported belowground and exudated from the roots within hours. These carbohydrates are mostly used by microbes and respired or converted into microbial biomass (Bahn et al., 2008; Bahn et al., 2009). In addition, Bahn et al. (2006) found that in mountain grasslands the heterotrophic component of soil respiration responds very spontaneously to changes in the availability of recent photosynthates. Autotrophic respiration on the other hand is buffered by large carbohydrate reserves in the extensive root system of high altitude plants, preventing fast responses to changing degrees of photosynthetic discrimination (Bahn et al., 2006). Thus, the dynamics observed in $\delta^{13}\text{C}_{\text{CO}_2}$ respired from the soil can be explained by the transition from carboxylation driven- to diffusion driven discrimination, reflected in the $\delta^{13}\text{C}$ signature of root exudates. Only the first values, late in April, are not consistent with the suggested mechanism. We assume that the highly enriched $\delta^{13}\text{C}_{\text{CO}_2}$ values, measured just after snowmelt, result from microbial cell lysis following winter freezing (suggested in Conen et al., 2008), making labile substrates available before the depleted, young C from the developing canopy becomes available as root exudates.

In the N deposition treatments, $\delta^{13}\text{C}_{\text{CO}_2}$ sampled in October 2010 (Fig. 3) were similar for N4_{control} and N14 ($-27.3\text{‰} \pm 0.07$ and $-27.5\text{‰} \pm 0.07$, respectively), but the value for N54 was higher ($-26.8\text{‰} \pm 0.05$). For comparison, $\delta^{13}\text{C}_{\text{SOC}}$ values in SOC of the $<1.4 \text{ g cm}^{-3}$ soil density fraction, equally sampled in October 2010, are approximately the same as those found in CO_2 : N4_{control} is $-27.0\text{‰} \pm 0.05$ and N14 is $-27.1\text{‰} \pm 0.05$, while N54 was less depleted ($-26.7\text{‰} \pm 0.08$). This suggests that the preferred substrates for soil respiration are contained in the $<1.4 \text{ g cm}^{-3}$ density fraction.

The amplitude of seasonal differences in $\delta^{13}\text{C}_{\text{CO}_2}$ in the high N54 deposition treatment was much smaller than that in the other N treatment levels. During summer, differences among N deposition treatments disappear. This is consistent with the assumption that soil CO_2 derives from respiration that is mostly fueled by very recent, labile carbohydrates from root exudates. Their isotopic ratios must be expected to converge towards a common, higher $\delta^{13}\text{C}$ when the isotopic fractionation is controlled by diffusion. This will be the case when the higher plant productivity found under N54 (Volk et al., 2014) leads to lower internal leaf CO_2 concentration and consequently to increased water use efficiency (Bassin et al., 2009; Dawson et al., 2002). As a result, isotopic fractionation in the N54 treatment is determined mostly by diffusion, instead of reflecting a fractionation process that is alternately determined by carboxylation or diffusion, as it is the case in the less productive N treatments.

4.4. Conclusion

Our results show the value of analyzing specific SOC pools when attempting to characterize and predict shifts in ecosystem-level C flux in response to atmospheric N-deposition, rather than treating SOC as a single compartment. The differentiated responses of density fractions result from complex interactions occurring in the soil, including possible priming effects,

which may trigger C-losses in individual fractions, even when overall SOC is increasing. We also confirmed that the isotopic signature of soil CO₂ originates mostly from the lightest soil fraction and can thus serve as a proxy for both seasonally/climate driven and N-deposition driven quality changes of the substrate used in soil respiration.

Clearly, our results show that it is inappropriate to use plant productivity N response as an indicator for shifts in SOC content in grassland ecosystems. Here, isotopic techniques helped to show that atmospheric N deposition of 14 kg N ha⁻¹ yr⁻¹ is below, and that 54 kg N ha⁻¹ yr⁻¹ is above a threshold that tips the balance between new, assimilative gains and respiratory losses towards a net loss of [SOC] for certain soil fractions in the subalpine grassland.

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Figure 1.

Linear regression of soil organic C concentration (C %) versus SOC $^{13}/^{12}\text{C}$ isotope mixing ratios ($\delta^{13}\text{C}_{\text{SOC}}$) for different soil density fractions and bulk soil at the start of the experiment (**A**) and after resampling seven years later (**B**). Soil density fractions are identified as: <1.4 (<1.4 g cm⁻³), <1.8 (1.4–1.8 g cm⁻³), >1.8 (1.8–2.2 g cm⁻³), >2.2 (>2.2 g cm⁻³). Values refer to the control treatment without additional N deposition (group means $\pm$ 1SE).

Figure 2.

Soil density fractions and bulk soil values for **A)** C concentration change (%C, positive values represent the absolute increase in C concentration observed in 2010) and **B)** C isotopic composition change (δ¹³C, negative values indicate the absolute increase in ¹³C depletion in 2010), after seven years of N deposition treatment. Groups of bars indicate the soil fraction indicated on the x-axis, shades of gray represent N deposition treatments N_{4control}, N₁₄ and N₅₄ (4, 14 and 54 kg N ha⁻¹ year⁻¹). Soil density fractions are identified as: <1.4 (<1.4 g cm⁻³), <1.8 (1.4–1.8 g cm⁻³), >1.8 (1.8–2.2 g cm⁻³), >2.2 (>2.2 g cm⁻³). Bars sharing the same lowercase letter within a group are not significantly different in planned comparison following one way ANOVA. All values are group means ± 1 SE.

Figure 3.

Soil respired $\delta^{13}\text{C}_{\text{CO}_2}$ during the 2010 snow-free period. Symbols indicate different N deposition treatments $\text{N4}_{\text{control}}$, N14 and N54 (4, 14 and 54 kg N ha⁻¹ year⁻¹). $\delta^{13}\text{C}_{\text{CO}_2}$ values sharing the same letter at individual measurement dates are not significantly different. All values are group means $\pm$ 1SE.

Table 1.

Soil organic carbon concentrations (% SOC), SOC $^{13/12}\text{C}$ isotope mixing ratios ($\delta^{13}\text{C}_{\text{SOC}}$) and corresponding P values of paired, two sided t-tests of soil density fractions and bulk soil in 2003 and after resampling in 2010. Values refer to the control treatment without additional N deposition.

Fraction	$\delta^{13}\text{C}_{\text{SOC}}$ (means $\pm$ 1 SE)				% SOC (means $\pm$ 1 SE)				P	
	2003		2010		2003		2010		$\delta^{13}\text{C}$	%C
<1.4	-25.93	(0.052)	-27.01	(0.047)	21.01	(0.594)	30.27	(0.818)	0.0000	0.0000
<1.8	-26.43	(0.032)	-26.86	(0.093)	25.09	(0.524)	27.48	(0.928)	0.0002	0.0198
>1.8	-25.76	(0.044)	-26.09	(0.059)	12.48	(0.252)	11.31	(0.239)	0.0003	0.0021
>2.2	-25.03	(0.035)	-25.23	(0.033)	5.02	(0.194)	3.98	(0.103)	0.0003	0.0001
bulk	-25.31	(0.033)	-25.56	(0.017)	8.51	(0.237)	10.09	(0.236)	0.0023	0.0001

Table 2.

Repeated measures ANOVA for N effects (N) and seasonal changes (Campaign) on $\delta^{13}\text{C}_{\text{CO}_2}$.

Term	DF	F-Ratio	P Level
N	2	3.9	0.038
Campaign	5	37.46	< 0.000
N $\times$ Campaign	10	1.37	0.212
Plot (N)	19		