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Carbon isotope discrimination during litter decomposition can be explained by selective use of substrate with differing $\delta^{13}C$

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Abstract

Temporal dynamics of C isotopic composition ($\delta^{13}C$) of CO_2 and leaf litter was monitored during a litter decomposition experiment using *Arbutus unedo* L., as a slow decomposing model substrate. This allowed us (1) to quantify isotopic discrimination variation during litter decomposition, and (2) to test whether selective substrate use or kinetic fractionation could explain the observed isotopic discrimination. Total cumulative CO_2-C loss (C_L) comprised 27% of initial litter C. Temporal evolution of C_L was simulated following a three-C-pool model. Isotopic composition of respired CO_2 (δ_{RL}) was higher with respect to that of the bulk litter. The isotopic discrimination $\Delta_{(L/R)}$ varied from −2‰ to 0‰ and it is mostly attributed to the variations of δ_{RL}. A three-pool model, with the three pools differing in their $\delta^{13}C$, described well the dynamic of $\Delta_{(L/R)}$, in the intermediate stage of the process. This suggests that the observed isotopic discrimination between respired CO_2 and bulk litter is in good agreement with the hypothesis of successive consumption of C compounds differing in $\delta^{13}C$ during decomposition. However, to explain also ^{13}C-CO_2 dynamics at the beginning and end of the incubation the model had to be modified, with discrimination factors ranging from −1‰ to −4.6‰ attributed to the labile and the recalcitrance pool, respectively. We propose that this discrimination is also the result of further selective use of specific substrates within the two pools, likely being both the labile and recalcitrant pool of composite nature. In fact, the 2‰ ^{13}C enrichment of the α-cellulose observed by the end of the experiment, and potentially attributable to kinetic fractionation, could not explain the measured $\Delta_{(L/R)}$ dynamics.

1 Introduction

Soils contain the largest terrestrial carbon (C) pool, and understanding the mechanisms controlling the flux of C is crucial to assess climate change and the potential C sink strength of soils (Jenkinson, 1991). In this context, stable C isotopes are very

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useful metabolic tracers to: (1) identify pathways and quantify rates of litter–C pools into different soil organic matter (SOM) pools (Balesdent et al., 1987; Bernoux et al., 1998; Hobbie et al., 2004; Rubino et al., 2007, 2010), (2) quantify and partition soil CO_2 efflux (Cheng, 1996; Wolfram et al., 2000; Ngao et al., 2005; Subke et al., 2006), and (3) link dissolved organic C (DOC) to respired CO_2 (Bengtson and Bengtsson, 2007). All these studies apply a linear mixing ratio between two (or more) sources and derive the contribution of two (or more) end-members to the pool or flux of interest using mass balance equations (Balesdent et al., 1987; Cheng, 1996). The mass balance approach generally relies on the assumption that there is no ^{13}C versus ^{12}C discrimination during heterotrophic respiration of organic matter (OM) substrates (Cheng, 1996; Ekblad et al., 2002; Ngao et al., 2005; Rubino et al., 2007). However, evidences exist that this assumption may be incorrect (Mary et al., 1992; Blagodatskaya et al., 2010). Uncertainties in the stable C isotopic composition ($\delta^{13}C$) of the C sources may significantly influence the estimates of the relative contribution of end members in mixing models (Philips and Gregg, 2001). Thus, understanding and quantifying isotopic discrimination during heterotrophic respiration and OM decomposition is necessary for accurately estimating belowground C input and CO_2 losses using isotopic methods.

Different theoretical models describing isotopic discrimination during decomposition of litter and SOM have been proposed (Ågren et al., 1996; Feng, 2002; Poage and Feng, 2004). Yet, we lack a full understanding and quantitative modeling of isotopic discrimination during heterotrophic respiration of the kind we have for photosynthesis (Farquhar et al., 1982). That is mainly because in the natural environment heterotrophic respiration arises from the breakdown of a composite substrate (e.g. plant tissue, soil organic matter), made of compounds (e.g. soluble sugars, lipids, structural carbohydrates, etc.) differing in their isotopic composition (Gleixner et al., 1993).

Two relevant processes may participate to differentiate the isotopic composition of the respired CO_2 from that of the OM substrate, during heterotrophic respiration: (i) selective use of C compounds with different $\delta^{13}C$, and (ii) kinetic fractionation, which results from enzymatic discrimination of ^{13}C versus ^{12}C (or viceversa) during the

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respiratory process. In the case of selective use, we would observe a difference in $\delta^{13}C$ between the product and the substrate, and this would not result from a true isotopic fractionation process during decomposition. Additionally, during soil respiration, also physical fractionation takes place in association to the diffusion of CO_2 between soil pore and the location where soil CO_2 efflux is measured, i.e. above the litter layer. In the soil, the latter has been well investigated and theoretical and experimental estimates are available (Amundson et al., 1998; Davidson, 1995; Kayler et al., 2010), thus we do not discuss physical fractionation in this study. We refer to isotopic discrimination (Δ) as to the difference in $\delta^{13}C$ between respired CO_2 (the product) and the OM which it is derived from (the substrate).

To identify whether a kinetic fractionation occurs during heterotrophic respiration, experiments have been conducted on individual substrates. Mary et al. (1992) pioneered this field demonstrating kinetic fractionation in heterotrophic respiration, but a mechanism for this observation is still not clear, and beyond the scope of the present study. Regardless of the type of discrimination (kinetic or selective use) during heterotrophic respiration, the quantification and mechanistic understanding of isotopic discrimination is crucial for the accurate use of natural abundance isotope technique for belowground C cycling work (Werth and Kuzyakov, 2010).

Several studies showed significant isotopic discrimination during microbial consumption of single compounds (Blair et al., 1985) and plant residues (Melillo et al., 1989; Schweizer et al., 1999; Connin et al., 2001; Kristiansen et al., 2004), but the results were highly variable, depending on the substrate and the organisms (bacteria, fungi) investigated. Only few studies looked at changes in $\delta^{13}C$ of both respired CO_2 and complex decomposing substrates (i.e. plant tissues) over time (Fernandez et al., 2003; Osono et al., 2008). And all of them suggest the selective use of C substrate as the main process driving isotopic discrimination during SOM decomposition, but do not present a model to explain those dynamics. Fernandez et al. (2003) suggested that plant quality may play a role in the observed C isotopic dynamics during early stages of litter decomposition, stages during which the lignin fraction have a limited influence

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on both mass loss and $\delta^{13}C$ of early-decomposing litter (Ngao et al., 2005; Osono et al., 2008).

The present study aims at: (1) characterizing the temporal dynamics of $\delta^{13}C$ of the CO_2 and of the leaf litter substrate from which it is respired, (2) quantifying isotopic discrimination (Δ) during litter decomposition and eventual variation of Δ over time, and (3) testing whether selective substrate use or kinetic fractionation can explain eventually observed isotopic discrimination. We hypothesized that (i) the $\delta^{13}C$ of respired CO_2 varies with time during litter decomposition and that, at any given time, it is different from the $\delta^{13}C$ of the bulk litter; (ii) at the study time scale, the variation in $\delta^{13}C$ of respired CO_2 is mainly explained by a selective use of C sources of different $\delta^{13}C$ and, thus, that (iii) no significant kinetic fractionation for a given C substrate, is expected. We performed a laboratory litter decomposition experiment using *Arbutus unedo* L. leaf litter, as a model composite substrate with a relatively low decay rates (Cotrufo et al., 2010b). A set of litter samples was incubated to follow the temporal evolution of respired CO_2 and its $\delta^{13}C$, while another larger set was incubated to characterize temporal evolution of chemical and isotopic composition of the remaining litter, by destructive samplings. We used cellulose as a model substrate to experimentally test the kinetic fractionation hypothesis. Our hypotheses were farther explored by model simulations.

2 Material and methods

2.1 Field litter sampling

The leaf litter was collected in a 50-ha coppiced stand dominated by the evergreen shrub *Arbutus unedo* (L.) at Tolfa-Allumiere (42°11′30″ N, 11°55′30″ E, 180 m a.s.l., Central Italy). For a full site description see Cotrufo et al. (2010a). At the site, *A. unedo* leaves fall in two main periods: May–June and October–November. Fully senesced *A. unedo* leaf litter was collected from standing trees in a strict *A. unedo*-dominated area

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in late May 2006, returned to the lab and litter was left to air dry. All leaves gathered together were assumed to represent a homogenous new fresh litter pool, as they were mixed well for several days during air drying.

2.2 Experimental setup

A. unedo litter samples were incubated in air tight mason jars filled each with 3–5 g of air-dried litter. A sub-set of air-dried litter was oven-dried at 70 °C in order to correct for the residual moisture. Before incubation, litter was sprinkled with deionized water to adjust moisture at 135% in gravimetric water content by weighting the jar. Airtight jars were incubated in the dark at 25°C. During the entire incubation period, litter water content was adjusted gravimetrically and headspace flushed. The first set of samples (Set 1, n=10) was dedicated to periodical CO_2 efflux and $\delta^{13}C$ of respired CO_2 measurements (see below). A second set (Set 2, n=70) was periodically harvested (n=5 at each harvest) for mass loss and chemical and isotopic analyses (see below).

2.3 CO_2 measurements

The incubation period (i.e. between two measurement dates) varied from 1 day at the beginning of the experiment to around 60 days by the end of it. The length of each incubation period was established in order to avoid excessive CO_2 enrichment and O_2 depletion in the jar headspace and to minimize the number of samplings. For each of the 10 replicate jars of Set 1, at the end of each incubation period, CO_2 concentration within the jar headspace was estimated by diluting a small amount of jar headspace air into a closed loop, which comprised an infrared gas analyzer (IRGA) Li-840 (Licor, Lincoln, USA) connected to a 0.5-L glass buffer, used to increase the total system volume (0.87 L). Before injection, the IRGA was left to run at a flow rate of 1 L min^{-1} in closed circuit, and a three-way connector placed at the IRGA outlet was left opened to the exit in order to reach both CO_2 concentration and pressure equilibrium (both were real-time monitored). Then, 0.02 L (V_{Syr}) of headspace air was sampled with a syringe

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and injected into the circuit. The syringe was removed and the three-way connector was closed, allowing pressure equilibration without any CO_2 concentration perturbation. The CO_2 concentration within the jar headspace (C_J, in ppmv) was calculated as:

$$C_J = V_{Syst}(C_{Final} - C_{Ini})V_{Syr}^{-1} \quad (1)$$

where V_{Syst} is the volume of the entire closed circuit (i.e. 0.87 L), C_{Ini} is the CO_2 concentration within the system before syringe injection (volume V_{Syr}), and C_{Final} is the CO_2 concentration within the system 1 m after syringe injection. Total respired C (C_L) is calculated by extrapolating C_J to the jar volume (1.37 L), assuming a molar volume of 24.79 L mol^{-1} and expressed on a litter dry mass basis (mg$_C$ g$_{DM}^{-1}$ d^{-1}).

Then after, the CO_2 accumulated during the incubation period within the jar headspace was cryogenically trapped. For this purpose, the jar was connected and opened to a cryogenic purification line (described by Bertolini et al., 2005). The headspace air was driven into the line at a constant flow rate of 0.1 L min^{-1} (mass flow controller Dwyer GFC 2104, Michigan City, USA) by a high vacuum pump (Mini-Task Varian Inc., Palo Alto, USA) placed at the end of the line. Along the line, first loop was bathed in an ethanol-dry ice mixture for scrubbing water vapor, and a second loop bathed in liquid nitrogen trapped CO_2 after setting the internal pressure at 250 mbar. Trapped CO_2 was transferred into a 6-mm PIREX® glass tube filled with reduced copper flakes. Cryogenic purification duration varied according to the CO_2 concentration within the jar determined as previously described. Afterwards, the jars were left opened for 30 min for CO_2 and O_2 equilibration as well as for stabilization of microbial activity. At this point, the instantaneous litter respiration rate was determined by measuring the increase rate of the CO_2 concentration within the jar for five minutes using the Li-840 IRGA in closed mode. This was done in order to estimate the length of the subsequent incubation period. All remaining background CO_2 within the jar headspace air was scrubbed for five minutes; so that exclusively litter respired CO_2 would accumulate during the following incubation period.

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2.4 Litter harvest, and chemical analyses

At each CO_2 measurement date, five replicate jars were harvested. Litter was oven–dried, weighed for mass loss and ground for chemical analyses. On the initial litter and on the litter samples collected at the end of the incubation experiment, α-cellulose was extracted from 0.2 g of ground litter enclosed in a porous bag (Filter bags, Ankom Technology, Macedon, USA) with two successive extractions using 5% NaOH solution for 2 h at 60 °C (Loader et al., 1997). The remaining material was washed 3 times with boiling distilled water, removing most of soluble compounds, fatty materials, resins, tannins and hemicelluloses. Then lignin was removed by a 36 h exctraction in a 7% sodium chlorite ($NaClO_2$) solution adjusted to a pH of 4–5 by adding 4–5 mL of 96% acetic acid (Loader et al., 1997), The remaining α-cellulose that was washed with boiling distilled water, oven-dried at 50 °C, weighted and prepared for isotopic analyses. Carbon concentration ($g\,C\,g_{DM}^{-1}$) of bulk litter and α-cellulose fraction were measured by an elemental analyzer (Flash EA 1112 NC, CE Instrument, Wingan, UK). All analyses were performed singularly on the five litter replicates.

2.5 Isotopic analysis

The $\delta^{13}C$ of the bulk litter (δ_{BL}) and the α-cellulose fraction (δ_{cell}) was measured by an elemental analyzer (Flash EA 1112 NC, CE Instrument, Wingan, UK) connected to an Isotope Ratio Mass Spectrometer (IRMS, Delta Plus, Thermo-Finnigan, Bremen, Germany). The calibration of the EA-IRMS setup was performed by analyzing the C6 ($\delta^{13}C = -10.80 \pm 0.47$‰; C content=42.13%) and C8 ($\delta^{13}C = -18.3$‰ ± 0.29‰; C content=26.67%) standard material provided by the International Atomic Energy Agency (IAEA, Vienna, Austria), as well as with an internal standard ($\delta^{13}C = -27.37 \pm 0.16$‰; C content=$8.93 \pm 0.89$%).

To eliminate N_2O, all CO_2 samples, enclosed in PIREX® tubes containing several grams of copper were preliminary baked at 400 °C during 30 min in order to reduce eventual N_2O into N_2 and CuO. This pre-treatment avoids overlapping in mass

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44 (confounding N_2O with $^{12}C^{16}O^{16}O$) and mass 45 (due to O^{17}) measurements. The $\delta^{13}C$ of each CO_2 sample (δ_{RL}) was determined by IRMS (Delta Plus, Thermo-Finnigan, Bremen, Germany) in Dual Inlet mode. The CO_2 samples from a first CO_2-in-air cylinder ($\delta^{13}C = -11.02 \pm 0.05$‰, certificated by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Atmospheric Research, Aspendale, Australia) and a second CO_2-in-air cylinder ($\delta^{13}C = -25$‰, Messer Griesheim GmbH, Krefeld, Germany) was cryogenized and analyzed for correcting fractionation occurring during the cryogenization step or instrumental biases. All $\delta^{13}C$ values are expressed against the international PDB standard.

2.6 Calculations and statistical analysis

All variables are expressed as daily rates calculated from either ten (CO_2 variables) or five (bulk litter material) replicates. A non–linear regression procedure (NLIN procedure, SAS v8, SAS Institute, USA) was used to fit the total litter CO_2-C loss (C_L, expressed as cumulated C loss relative to initial litter C amount, in $g_C\ g_{ini}^{-1}$) over time (t) to a multi-pool single exponential model according to:

$$C_L(t) = \sum_i f_i \left(1 - e^{-k_i t}\right) + \varepsilon \quad (2)$$

where f_i is the fraction of a C pool i participating to C_L and k_i is the decomposition rate constant of the respective C pool (d^{-1}), and ε a residual term. We compared the Eq. (2) for either i=2 (two-pool model) or i=3 (three-pool model) by calculating for each model the Akaike Information Criterion, corrected for small samples (AIC_C, McQuarrie and Tsai, 1998):

$$AIC_C = \ln\left(RSS/(n-p)\right) + (n+p)/(n-p-2) \quad (3)$$

where n is the number of samples, RSS is residual sum of square and p is the number of model parameters. The lowest AIC_C value indicates the best model.

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In order to explore the causes of the variations of δ_{RL} over time, we first derived the fractional contributions of each of tree pools (f_{C_i}):

$$f_{C_i}(t) = f_i \left(1 - e^{-k_i t}\right) / C_L(t) \quad (4)$$

with the f_i and k_i parameters and C_L values from the Eq. (2). Then, a mass balance approach was adopted in order to test if the $\delta^{13}C$ of the different pools weighted by their fractional contribution could reproduce the $\delta^{13}C$ of litter CO_2-C loss (δ_{RL}) according to:

$$\delta_{RLsim}(t) = \sum_i f_{C_i}(t)\delta_i(t) \text{ and } \sum_i f_{C_i}(t) = 1 \quad (5)$$

where δ_i is the $\delta^{13}C$ the C pool i. Moreover, Eq. (5) assumes no direct fractionation on the i pool (i.e. $\delta_i = \delta^{13}C$ of CO_2 derived from the i pool).

C isotopic discrimination ($\Delta_{(L/R)}$) between litter OM and respired CO_2 was calculated as:

$$\Delta_{(L/R)} = (\delta_{BL} - \delta_{RL}) / (1 + \delta_{RL}) \quad (6)$$

where $\delta^{13}C_{BL}$ is the $\delta^{13}C$ of the remaining litter material. Linear regression analyses were performed to calculate the CO_2 concentration rate (ppmv s^{-1}), hereafter converted in litter respiration rate (in µmol g_{DM}^{-1} s^{-1}). The $\delta^{13}C$ values of the different C components (respired CO_2, bulk litter, α-cellulose) were averaged per sampling day. One-way ANOVAs analyses were used to compare daily $\delta^{13}C$ mean values among the different C components by date (STATGRAPHICS Plus 4.1).

3 Results

3.1 Litter respired CO_2

Litter respiration rate was high (around 2 mg C g_{DM}^{-1} d^{-1}) at the beginning of the incubation, reduced by half in the following two months to decline slower until the 4th

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month (day of incubation DOI 137), where it remained stable until the end of the experiment (Fig. 1). Very little variation was observed between replicate litters (standard error (n=10) not exceeding 0.0002 mg C g_{DM}^{-1} d^{-1}). The total cumulated C_L was 457 ± 53 mg C y^{-1}, corresponding to around 27% of initial litter C. Temporal evolution of C_L (Fig. 2) was modeled following Eq. (2) (R^2>0.999; rmse=0.003; p<0.0001) using three C pools (i=3). Adding a third pool enhanced significantly the description of dynamics of CO_2-C loss, as compared to a two-pool model (i=2; R^2=0.998; rmse=0.005; p=0.0001), with the AIC_C value (AIC_C=−9.918) being lower for the three- than for the two-pool model (AIC_C=−8.271). The three C pools contributed at different proportions to the total litter respired CO_2, and had significantly different decomposition rate constants k (Table 1). The fractional contribution of each C pool, calculated according to Eq. (4) varied with time. The labile pool was the main contributor during the first 70 days while after the intermediate pool became the main contributor to litter CO_2-C loss (Fig. 2).

3.2 Remaining litter

Mean litter mass-C loss averaged 27% of initial mass-C over one year. Mass-C loss and CO_2-C loss were very close in daily averages (R^2=0.95, p<0.001, Fig. 3), allowing us to assume that (1) C mass loss exclusively occurred by microbial CO_2 production as opposed to either fragmentation or leaching, and (2) the two sets decomposed in a similar way. The extracted α-cellulose concentration ranged from 13.80 ± 0.02% to 5.72 ± 1.37% of total dry matter (Fig. 4).

3.3 Carbon isotopes

3.3.1 Temporal dynamics

The $\delta^{13}C$ of litter respired CO_2 (δ_{RL}) increased significantly during the first 3 days of incubation by about 1‰, and decreased to −29.36 ± 0.38‰ at the end of incubation.

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In contrast, the $\delta^{13}C$ of remaining bulk litter (δ_{BL}) did not vary significantly throughout the experiment, averaging -29.14 ± 0.01‰ (Fig. 5). Thus, the C isotopic discrimination $\Delta_{(L/R)}$ ranged from −2‰ to 0‰ (Fig. 5), and it is attributable to the variations of δ_{RL}. The δ_{RL} values were positively correlated to litter α-cellulose concentration values (Fig. 6). The $\delta^{13}C$ of α-cellulose (δ_{cell}) averaged -28.81 ± 0.25‰ in initial litter samples, while it averaged -26.74 ± 0.16‰ in the most advanced decomposing litter samples, highlighting a significant ^{13}C enrichment of α-cellulose during decomposition (p=0.001, Fig. 5) by around 2‰.

3.3.2 Model simulations

To test for the selective use of substrate hypothesis, we constrained the mass balance Eq. (5) by assuming no kinetic discrimination on any of the three pools, with δ_1 being equal to −29.09‰, the measured $\delta^{13}C$ of the remaining bulk litter at the end of the incubation period – the recalcitrant pool, δ_2 being equal to −25.5‰, as a mean $\delta^{13}C$ of soluble compounds – the labile pool – (Schweizer et al., 1999), and δ_3 as the initial $\delta^{13}C$ of α-cellulose (i.e. −28.81‰). As result of this simulation, the computed δ_{RL} (δ_{RLsim1}) were in very good agreement for a period between DOY 66 and 227, during which the intermediate pool is the major contributor to total litter CO_2-C loss (Fig. 7). This result suggested that the observed $^{13}CO_2$ dynamics can be explained by a three-pool decay model with the three pools having different C isotope ratios, and that the α-cellulose could be used as a proxy for the intermediate pool. However, it fails to explain the $^{13}CO_2$ dynamics at the beginning (DOY 0–65) and end (DOY 228–365) of the incubation period (Fig. 7), suggesting either that kinetic discrimination may be occurring, or that the labile and recalcitrant pools are composite, in terms of the $\delta^{13}C$ of the specific compounds that contribute to those pools.

To explore if kinetic fractionation could explain the discrepancy between δ_{RL} and δ_{RLsim1}, the measured change in $\delta^{13}C$ of α-cellulose was incorporated to the Eq. (5), assuming a linear interpolation between the two mean values (i.e. constant kinetic

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fractionation), with the other δ_i values (recalcitrant and labile) being the same as above. Taking into account the measured cellulose ^{13}C-enrichment actually led to a larger discrepancy between the computed δ_{RL} (δ_{RLsim2}) and the measured δ_{LR} (Fig. 7).

As an alternative test, we modified the δ values of the labile and recalcitrant pool by introducing a discrimination factor (a_i) into the Eq. (5), which lead to the following equation:

$$\delta_{RLsim3} = f_{C1}(\delta_1 + a_1) + f_{C2}(\delta_2 + a_2) + f_{C3}(\delta_{cell}) \quad (7)$$

with the δ_i values being the same as above. We used Eq. (7) to compute δ_{RL} (δ_{RLsim3}, Fig. 7). Between DOY 1 and 31, the δ_{RLsim3} was found to be sensitive to the a_2 (i.e. of the labile pool), while between DOY 308 and 371, the model was sensitive to a_1 (i.e. of the recalcitrant pool). We found that δ_{RLsim3} best matched δ_{RL}, when a_1 was set at −1‰ between DOY 1 and 4 and at −0.5‰ between DOY 8 and 31, and when a_2 was set of at −4.6‰ between DOY 308 and 371, while in all other period both a_1 and a_2 were set equal to 0 (Fig. 7).

4 Discussion

Isotopic discrimination during CO_2 production has been observed in several studies involving consumption of composite substrate such as plant tissues (Schleser et al., 1999; Schweizer et al., 1999; Fernandez et al., 2003; Kristiansen et al., 2004), soil organic C (Šanctrůčková et al., 2000) or SOM (Crow et al., 2006; Blagodatskaya et al., 2010). In two of these studies and despite the difference in level of complexity of the involved substrates, the δ^{13}C of respired CO_2 was initially depleted with respect to initial bulk material and evolved with time through more ^{13}C-enriched CO_2 (Fernandez et al., 2003; Crow et al., 2006). In our study, we found the opposite temporal evolution, as shown by the $\Delta_{(L/R)}$ varying from −2‰ to 0‰. This contradictory result may be partly explained by the high lignin content of *A. unedo* leaf litter (35.85%, Piermatteo, 2007), as well as being a tannin-rich species (Rogosic et al., 2006). These secondary

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compounds are known to be generally strongly ^{13}C-depleted towards water soluble carbohydrates of the same origin (Gleixner et al., 1993) consumed during the early stages of decomposition (Ngao et al., 2009). Also this would explain the rather ^{13}C-depleted signal of our initial bulk litter (−29.05‰) compared to respired CO_2 (Fig. 5) or labile C compounds such as root and stem phloem sucrose (which are enriched by about 5‰ in *A. unedo* trees from the same site, data not shown).

Most of the δ^{13}C-based studies have attributed the origin of isotopic discrimination of soil CO_2 efflux or during litter decomposition to either a selective use of C substrates differing in δ^{13}C or to kinetic fractionation during microbial breakdown of specific compounds. But, to date, no consensus has been reached as, for example, shown by the contradictory conclusions of Boström et al. (2007) and Rubino et al. (2007). Different studies found evidences in support of the selective use of substrate as the mechanism of isotopic discrimination during litter decomposition. Fernandez et al. (2003) and Crow (2006) showed significant correlations between the initial respired CO_2 (during which Δ is negative) and the labile C content of composite residues. Recently, Blagodatskaya et al. (2010) conducted soil incubations with C3- and C4-planted soil, and they concluded that the preferential use of different substrates drove isotopic discrimination among SOM, microbial C and respired CO_2, microbial fractionation being neglegible.

In our study, we showed that during decomposition of δ_{RL} varied over time and that this variation can be partly explained by a three pool model (i.e. a labile, an intermediate stable and a recalcitrant pool), where the pools differentially contribute to litter decomposition and differ in their δ^{13}C. Moreover, the δ_{BL} did not significantly vary, likely because the relatively small total C loss (maximum of 27% of initial), coupled with a relatively small variation in the δ^{13}C of the three pools (within 3.5‰) did not make the changes in δ^{13}C of the remaining C appreciable. Thus, the $\Delta_{(L/R)}$ varied due to changes in $\delta^{13}C_{RL}$. In addition, it was found that δ_{RL} was significantly correlated to the concentration of α-cellulose, which represented around 15% of the total initial *A. unedo* litter dry mass, indicating that the cellulose C fraction is a major driver of the temporal evolution of δ_{RL}.

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Temporal evolution of litter respired CO_2 was best described using a three-pool model (Andren and Paustian, 1987). Several authors modeled their cumulated CO_2 dynamics using a two-pool model (Fernandez et al., 2003; Rubino et al., 2007), but in our case, adding a third pool improved significantly the explicative efficiency of the model. The C_L evolution revealed three kinetic C pools with significantly different k values (in the order of those found by Couteaux et al. (1998)). The three-pool model was used to derive the fractional contribution of each pool, allowing building an isotopic mass balance for testing to which extent the preferential use of C pools drove the δ_{RL} dynamics (Eq. 4; Fig. 7), assuming that the three pools where unique with distinct and constant $\delta^{13}C$ values. This approach revealed that the observed isotopic discrimination could be explained only during the intermediate phase of decomposition, when the intermediate pool makes the largest fractional contribution to litter respiration (Fig. 2), thus suggesting that the only pool that could be considered unique was indeed the intermediate pool which in our case is well described by the α-cellulose. In fact, when the $\delta^{13}C$ values of the labile and recalcitrant pools were allowed to change (Eq. 7) the simulated δ_{RLsim3} better matched the measured values, suggesting that either some kinetic fractionation was occurring or that those two pools were not unique, and that within them preferential use of substrates with different $\delta^{13}C$ was taking place. Further experimental work needs to be done to clarify the above results. We offer here our interpretation based on data and speculation.

(1) The labile pool is likely a composite pool, made of dead microbial biomass C, sugars, organic acids and other soluble organic compounds, easily consumed by microbes (Cadish and Giller, 1997; Ngao et al., 2009). We originally assumed that the $\delta^{13}C$ of the labile pool (δ_2) is unique and corresponds to that measured for litter soluble compounds by Schweizer et al. (1999). Indeed this assumption may be biased because small $\delta^{13}C$ differences may occur among soluble compounds extracted from different litter and because the chosen $\delta^{13}C$ value of the labile pool (−25.5‰) is likely more similar to that of microbial C than of water-soluble C (lower to ca. 1–2‰), as determined in a C3 soil by Pelz et al. (2005) in agreement with Śanctrůčková et al. (2000),

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which would contribute to respiration at the very early stage of litter decay. Damesin and Lelarge (2003) measured the $\delta^{13}C$ of sugars of newly fallen leaves (i.e. fresh leaf litter) to be around −28‰. When we modified our model and assumed, for the initial decay period, a $\delta^{13}C$ of the labile fraction 1‰ to 0.5‰ lower that that used successively, the computed δ_{RLsim3} better matched the measured δ_{RL} (Fig. 7). We therefore suggest that the labile fraction is a composite pool – of possibly 3 sub-fractions with slightly different $\delta^{13}C$, and with the relatively most ^{13}C-depleted compounds being respired earlier in the decomposition process.

(2) We assumed that the $\delta^{13}C$ of the intermediate pool is a unique pool which corresponds to the $\delta^{13}C$ measured for α-cellulose on the initial litter sample. This assumption allowed to successfully simulating the δ_{RL} for the period during which the intermediate pool is the main contributor to C_L. Moreover, this assumption was also supported by the similarity in the relative size of the modeled intermediate pool (f_3=16.80%, Table 1) and that of the α-cellulose in the initial litter (13.80%, Fig. 4). The observed ^{13}C-enrichment of the α-cellulose during decomposition does not explain the observed $\Delta_{(L/R)}$ dynamics and, thus, we suggest that kinetic fractionation even if it may occur (see below) is not the main mechanism behind isotopic discrimination during litter decomposition. As mentioned previously, further work has to be done to better describing this change of $\delta^{13}C$ to better integrate it within our model

(3) We initially assumed that the recalcitrant C pool is a unique fraction and that its $\delta^{13}C$ corresponds to the $\delta^{13}C$ of the remaining litter. However, as for the labile pool, introducing for the recalcitrant pool a discrimination factor in Eq. (7) of −4.6‰ between DOY 308 and 371, allowed to successfully simulating the δ_{RL} (δ_{RLsim3}, Fig. 7). This discrimination factor is consistent with Schweizer et al. (1999) that reported lignin values which are ^{13}C-depleted by 4.6‰ with respect to cellulose in C3 leaves (see also the references in Hobbie and Werner (2004) for lignin values in plant materials). In light of those findings, we suggest that also the recalcitrant pool is a composite pool, with the more ^{13}C-depleted lignin component contributing the majority of this pool at the latest stage of decomposition.

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Our results suggested that temporal δ^{13}C-CO_2 dynamics can be explained by the selective use of different C pools with different δ^{13}C varying over time. But it also highlighted that while three pools are enough to describe leaf litter decay rates, more substrates with distinct δ^{13}C are needed to describe $\Delta_{(L/R)}$ dynamics, at least during the initial and late stage of decomposition.

Indeed, we also observed a ^{13}C-enrichment of α-cellulose extracted from the decomposing litter over time. This result questioned the validity of assumption that the constant δ^{13}C of the C pools used for the mass balance equations (i.e. Eq. 5), and opens to the possibility of kinetic fractionation during consumption of single substrate. However, as said above, at our experiment time scale, the 2‰-enrichment of α-cellulose throughout litter decomposition is in the same order of magnitude of the ^{13}C-enrichment of litter respired CO_2, and thus it may not explain the observed $\Delta_{(L/R)}$ dynamics.

Although it is possible that isotopic fractionation occurred during the cellulose extraction, all our samples were processed altogether in the same NaOH and $NaClO_2$ baths, making it unlikely. Tannin–proteins complexes also can be produced during decomposition (Preston et al., 1997) and influence the δ^{13}C of bulk litter but were removed during the NaOH digestion (Loader et al., 1997). With regards to the kinetic discrimination during microbial respiration of individual C pools, several authors showed an isotopic fractionation against ^{13}C during microbial respiration of a single C substrate such as glucose, leading to depleted respired CO_2 (Blair et al., 1985; Mary et al., 1992; Šanctrůčková et al., 2000) or not (Ekblad et al., 2002). Schleser et al. (1999) reported isotopic discrimination during thermal decomposition of wood cellulose. However, to our knowledge, this is the first study that measured the δ^{13}C of a polymerized substrate over time, and observed a variation during litter incubations. Contrary to Engelking et al. (2007), this result suggests that some kinetic fractionation process might have also taken place during the decomposition of cellulose. We can identify three possible ways by which this fractionation had occurred, which do not exclude each other:

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1. A carbon isotopic fractionation of the cellulase complex, which may promote a preferential use of "light" parts of α-cellulose by microorganisms. This effect could originate from intra-molecular heterogeneity in ^{13}C within the cellulose, as a consequence of discrimination during photosynthetic and post-photosynthetic processes, and the production of secondary compounds (Hobbie and Werner, 2004). To our knowledge, there is no information on such kinetic fractionation of any parts of this enzymatic complex (endo- and exo-glucanase, β-glucosidase) during the disruption of the crystalline structure followed by depolymerization, as shown for the Krebs cycle (Blair et al., 1985; Geissler et al., 2009).

2. A preferential release of $^{12}CO_2$ versus $^{13}CO_2$ during cellulose breakdown was already shown by Blair et al. (1985), An isotopic fractionation related to CO_2 production by the microbial biomass, was also evidenced by Blagodatskaya et al. (2010) but it was very minor with regard to the preferential use effect.

3. An isotopic discrimination due to microbial community shift during cellulose decomposition, of the kind that was shown by Haichar et al. (2007) for bacteria. Moreover, Henn and Chapela (2000) showed that the fungal community structure may induce an isotopic discrimination when trioses are assimilated after extracellular cellulose digestion or not when glucose is directly assimilated.

Clearly the transformations of C compounds during decomposition need to be better described to constrain isotopic-based models, improving the knowledge of microbial isotopic discrimination and further applications in field partitioning of soil CO_2 efflux.

5 Conclusions

Litter respired CO_2 was ^{13}C-enriched respective to the bulk litter material. This discrimination was shown to be related to a selective use of C sources of different $\delta^{13}C$. Modeling the temporal variation of litter decomposition through CO_2-C loss favored

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a time-varying fractionation of (at least) three different pools to the temporal variation of respired CO_2. Our data were best described by an isotopic mass balance of such model including the parameters of remaining litter material, suggesting that the isotopic discrimination observed between respired CO_2 and bulk litter is mainly driven by selective consumption of various C compounds differing in $\delta^{13}C$ and in decay rates. However, while three pools well explain litter decay dynamics, more pools with slightly distinct $\delta^{13}C$ appear to be needed to account for $\Delta_{(L/R)}$ dynamics, in particular at the initial and late stages of litter decomposition. Additionally, our study showed an increase of the $\delta^{13}C$ of decomposing litter α-cellulose, but proved that kinetic fractionation of this pool alone cannot explain the observed $\Delta_{(L/R)}$ dynamics. These results confirm that isotopic discrimination during decomposition of litter and other composite OM substrates needs to be taken into account to prevent biases in determining soil C dynamics by natural abundance stable C isotopes (Werth and Kuzyakov, 2010), which can be corrected for by using the proposed approach.

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Table 1. Values of fractional proportions (f_i, unitless) and decomposition rate constants (k_i, d^{-1}) fitted from the three-C pool model (Eq. 2). For constraining Eq. (4), δ_1 was set equal to the measured $\delta^{13}C$ of remaining bulk litter (δ_{BL}), δ_2 was set equal to −25.5‰ (Schweizer et al., 1999), and δ_3 was set at −28.81‰, i.e. the $\delta^{13}C$ of cellulose in initial litter.

C pool	f_i	k_i	δ_i
1 – Recalcitrant	0.776	0.0002	δ_{BL}
2 – Labile	0.061	0.0635	−25.5
3 – Intermediate	0.163	0.0067	−28.81

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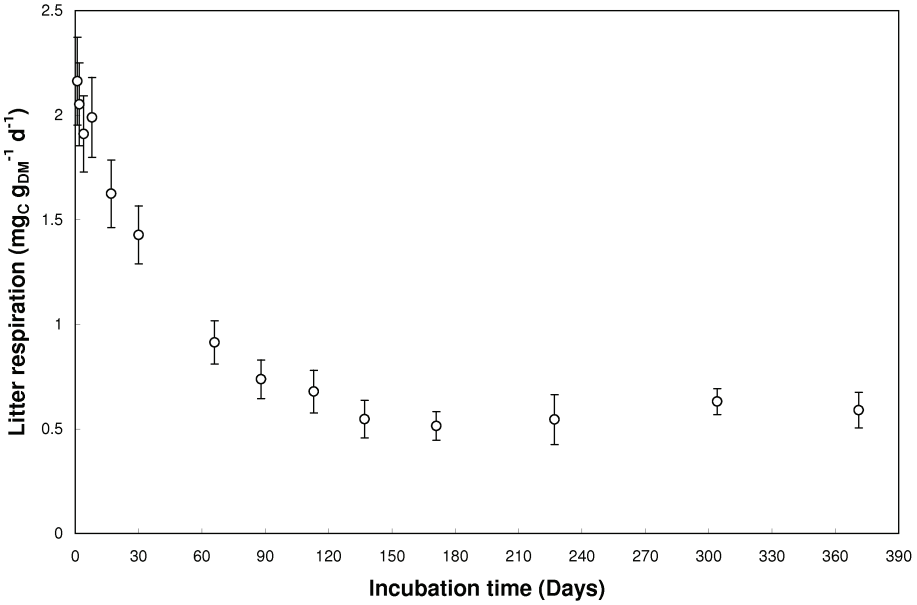

Fig. 1. Temporal evolution of mean litter respiration rate (± standard errors , n=10).

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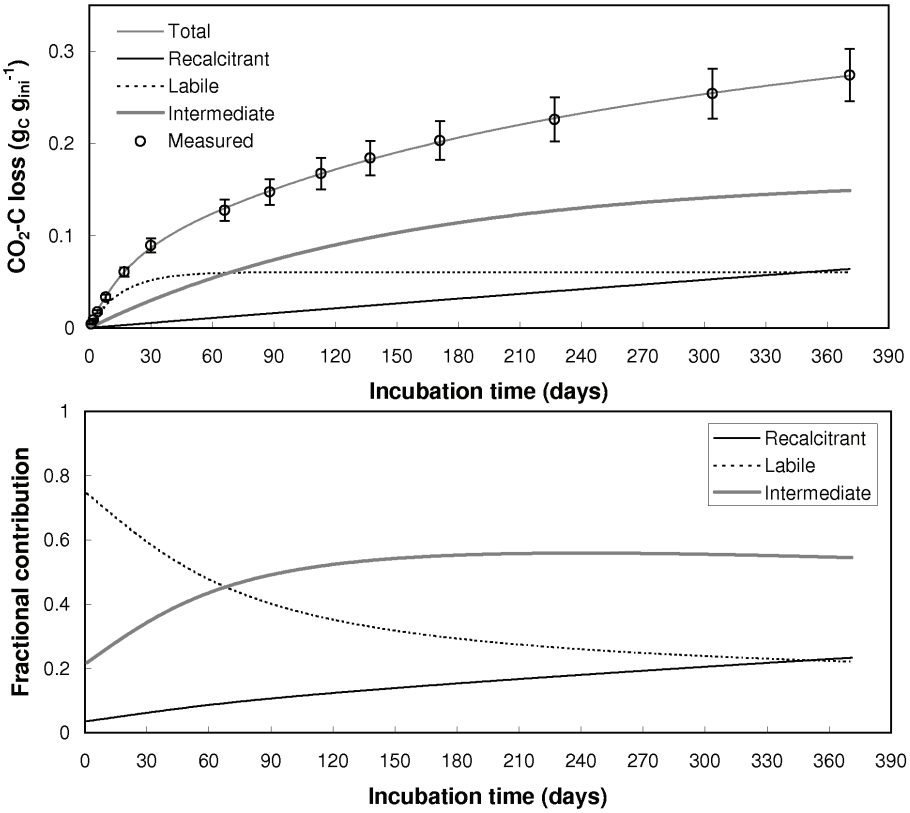

Fig. 2. Upper panel. Temporal evolution of cumulative respired CO_2-C (C_L in the text) expressed as fraction of initial litter C (open circle, mean ± standard errors). Temporal evolution of total cumulative respired CO_2-C (solid grey line) and that of the three C pools were simulated according to Eqs. (2) and (4), respectively (see text for details, see Table 1 for model parametrisation). Lower panel. Temporal evolution of fractional contribution (unitless) to total litter cumulated CO_2-C of each C pool as calculated by Eq. (4).

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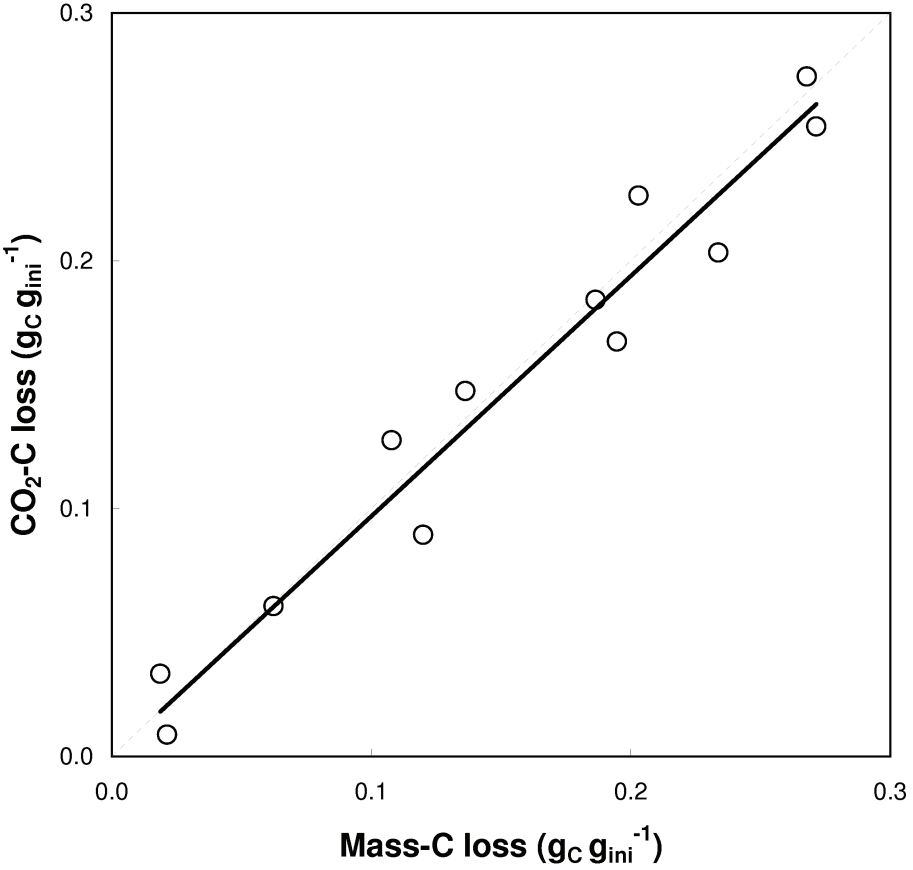

Fig. 3. Comparison between cumulated CO_2-C loss (C_L in the text) estimated by CO_2 concentration measurements and mass-C loss by litter harvests, both expressed as fraction of initial litter C ($g_C\ g_{ini}^{-1}$). The CO_2-C and mass-C fractions were linearly related such as CO_2-C=0.9693 mass-C (R^2=0.95, p<0.001).

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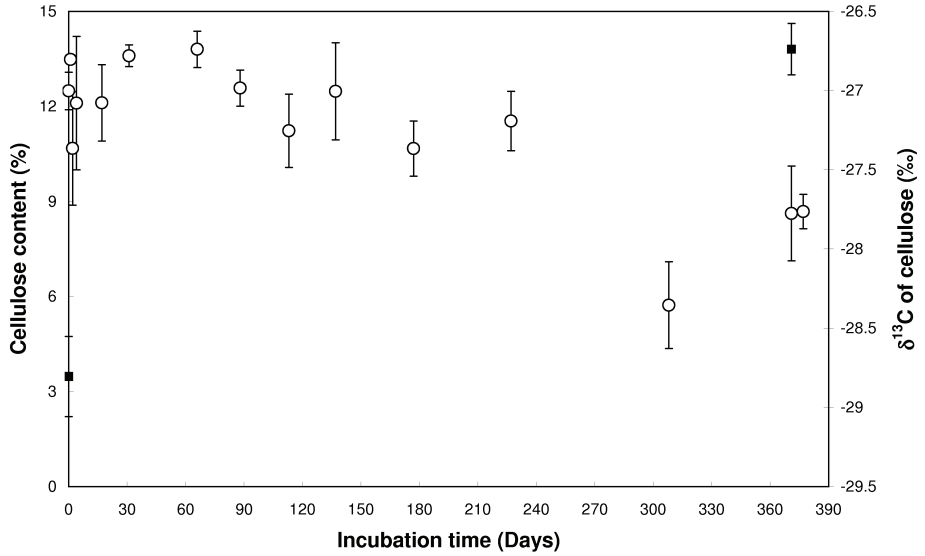

Fig. 4. Temporal variation of α-cellulose content (open circle) and of its isotopic composition (δ^{13}C, closed squares). Error bars are standard errors.

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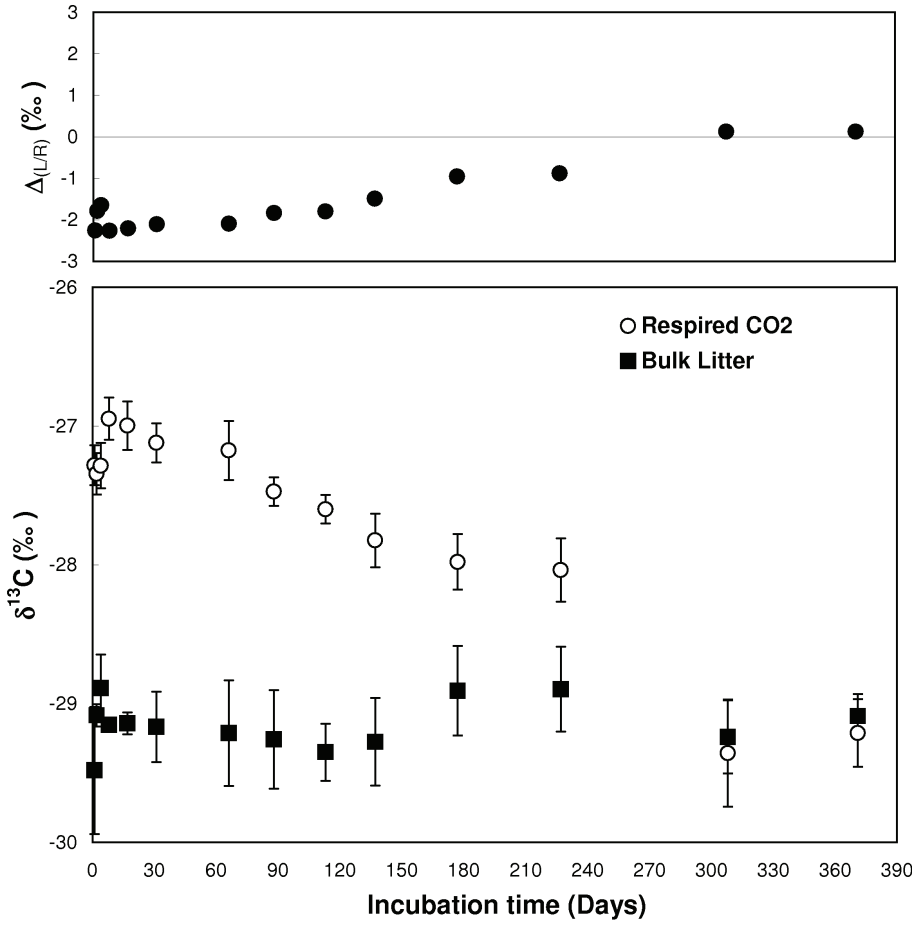

Fig. 5. Lower panel: Temporal evolution of mean $\delta^{13}C$ of respired CO_2 (open circles, n=10) and bulk litter organic matter (closed squares, n=5). Upper panel: Temporal evolution of isotopic discrimination ($\Delta_{(L/R)}$) between respired CO_2 and bulk litter. Error bars are standard errors.

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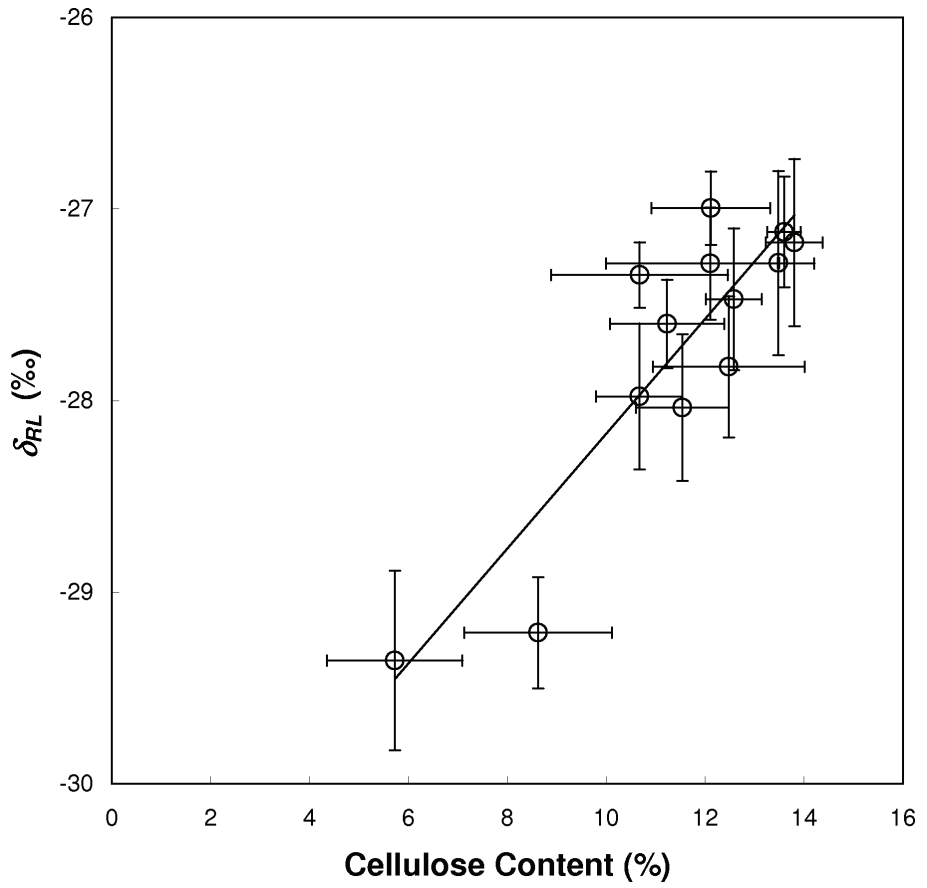

Fig. 6. Relationship between remaining cellulose content and $\delta^{13}C$ of litter respired CO_2 (δ_{RL}). The error bars are standard errors. The solid line is the linear regression as: δ_{RL}=0.3 (Cellulose)–31.17 (R^2=0.78, p<0.05).

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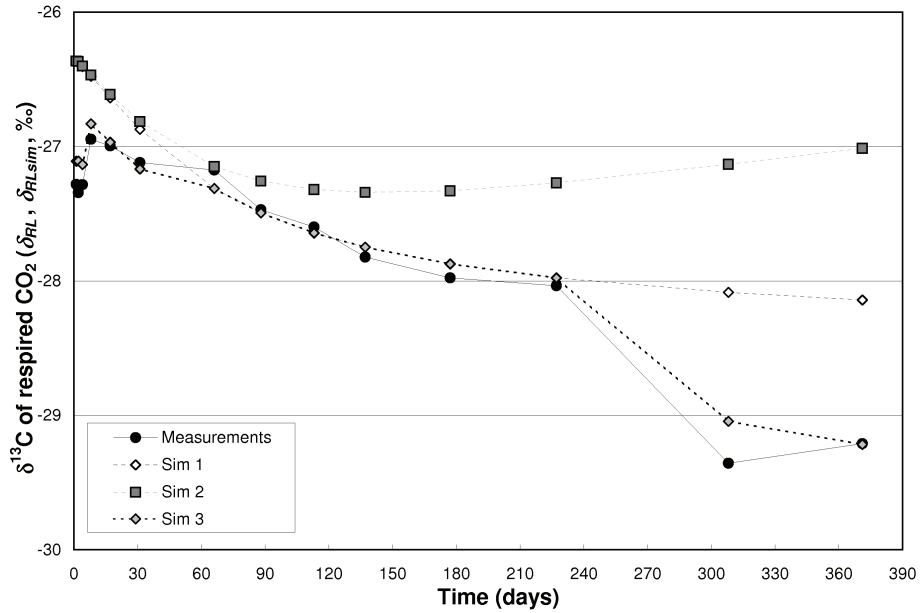

Fig. 7. Time-course of the measured (Mes, δ_{RL}, circles) or simulated (Sim, δ_{RLsim}, diamonds) $\delta^{13}C$ of litter CO_2-C loss as computed by Eq. (5) (see the text for more details). The simulation Sim1 was performed by applying directly the Eq. (5) with the f_{C_i} and δ_i parameters as detailed in the text, as well for the simulation Sim2 which takes also into account the change in $\delta^{13}C$ of the cellulose used as a proxy of the intermediate pool. The simulation Sim3 was the same as Sim1, plus accounting in Eq. (5) a discrimination factor of −1‰ on the labile pool at t=DOY 1 (a_1 in Eq. 7), and discrimination factors of −4.6‰ for recalcitrant pools (a_3, Eq. 7) at t=DOY 371.

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