Palatability trials on hardwood leaf litter grown under elevated CO$_2$: a stable carbon isotope study

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Received 1 April 2004; received in revised form 12 November 2004; accepted 15 November 2004

Abstract

The palatability to isopods and microbes of a broad range of hardwood leaf litter, derived from three field CO$_2$-enrichment experiments in the USA, was investigated, using $\delta^{13}$C, to trace the C flow from litter to isopods and to CO$_2$ respired by microbial decomposition. Leaf litter grown under elevated CO$_2$ had $\delta^{13}$C values ranging from $-39$ to $-45\%$, which were significantly different from ambient litter $\delta^{13}$C values of around $-30\%$. Litter palatability to isopods of the Porcellio sp. was tested by incubating ambient- and elevated-CO$_2$ litter, and a mixture of the two, in the presence of isopods for 14 days, under environmentally controlled conditions; $\delta^{13}$C was measured on litter and isopods’ body before and after incubation. In an additional experiment, litter was incubated in the absence of fauna for 30 days, and on five occasions the $\delta^{13}$C of the CO$_2$ respired from litter was measured. The $^{13}$C label was clearly carried from the litter source to the isopods’ bodies, and their faeces. For microbial-respired CO$_2$, $\delta^{13}$C was significantly higher than that of the litter source, suggesting preferential degradation of substrates enriched in $^{13}$C as compared to those in the overall litter. With the exception of Quercus myrtifolia leaf litter, elevated CO$_2$ did not affect the palatability to isopods nor the microbial degradation of any of the litters, possibly as a result of unaltered litter N concentration. However, significant differences in litter palatability and decay rates were observed among the different species. With this study, the use of isotopically labelled litter material was confirmed as a key methodology that can significantly contribute to the advancement of the understanding of litter decomposition and of the quantification of C fluxes in the process.

Keywords: Stable C isotopes; Elevated CO$_2$; Leaf litter; Porcellio sp.; Decomposition

1. Introduction

Plant tissues generated under elevated atmospheric carbon dioxide ($^{4}{\text{CO}}_2$) are often of a different quality than tissues generated under ambient $^{4}{\text{CO}}_2$. In particular, they can show a decrease in nitrogen (N) concentrations (Cotrufo et al., 1998a); a general, but not statistically significant, increase in lignin concentration (Norby et al., 2001a); a change in the composition and concentration of secondary plant metabolites (Peñuelas et al., 1997); a modification of their physical structure, with thicker leaves (Vu et al., 1989). On the basis of these observations, it has been hypothesised that plant tissues generated under elevated $^{4}{\text{CO}}_2$ would be less palatable to insects (Roth and Lindroth, 1995). This hypothesis, however, can result in two opposite responses: (1) insects would consume a higher quantity of plant tissues generated under elevated $^{4}{\text{CO}}_2$, because of their poorer nutritional value (higher C/N ratio) to satisfy their needs; (2) insects would avoid feeding on the less nutritious tissues, with all the implications and feedbacks that this change may generate at the community level.

In order to investigate if plant tissues generated under elevated $^{4}{\text{CO}}_2$ are less palatable to insects and also to microbial decomposers, and to determine which of the two above responses is more likely to occur, experiments must be designed such that the absolute palatability (i.e. consumption) and preferential use of ambient and elevated $^{4}{\text{CO}}_2$ tissues can be assessed. A powerful technique to study food webs and C flows between resources and feeders is...
the use of C isotope tracing (Radajewski et al., 2000). The variation in the natural abundance of $^{13}$C was used in several studies of faunal assimilation of organic matter (Martin and Lavelle, 1992) as well as indicator of substrate use by microbial communities (Abraham et al., 1998). Most CO$_2$-enrichment experiments use CO$_2$ derived from the combustion of fossil fuels, which is depleted in $^{13}$C with respect to the background $^{13}$CO$_2$. Therefore, these are labelling experiments generating plant tissues depleted in $^{13}$C concentrations. This approach to C labelling has found many successful applications in the study of C cycling (Ineson et al., 1996; Van Groenigen et al., 2002) and additional application are envisaged (Pataki et al., 2003).

The current experiment was the first attempt to use $\delta^{13}$C to trace the effects of elevated $^{13}$CO$_2$ on C flow from litter to isopods and to CO$_2$ respired by microbial decomposition. We used a broad range of litters derived from three field CO$_2$-enrichment experiments in the USA. Isopods were chosen for the trial since they are among the main groups of macroarthropods that contribute to plant litter breakdown (Dickinson and Pugh, 1974), and have been previously used to assess the impact of elevated CO$_2$ on litter palatability (Cotrufo et al., 1998b; Hättenschwiler et al., 1999; David et al., 2001).

2. Material and methods

2.1. Litter samples

Hardwood leaf litters derived from CO$_2$-enrichment experiments, where the CO$_2$ used for fumigation was derived from fossil fuel and thus depleted in $^{13}$C, were used. The CO$_2$ fumigation study sites were:

1. Free air carbon dioxide enrichment (FACE) experiment in the Duke Forest (Orange County, North Carolina, USA), with six experimental plots (30 m diameter), three maintained at ambient $^{13}$CO$_2$ and three constantly enriched with 200 $\mu$L L$^{-1}$ CO$_2$ above ambient (Hendrey et al., 1999). From this site, litter of Pinus taeda; Liquidambartyraciflua, Acer rubrum, and Cercis canadensis was sampled.

2. Oak Ridge FACE experiment at Oak Ridge National Laboratory, TN, USA. At this site, L. styraciflua leaf litter was collected from the four experimental plots (25 m diameter), two at ambient $^{13}$CO$_2$ and two enriched with a CO$_2$ concentration set point of 553 $\mu$L L$^{-1}$ CO$_2$ (Norby et al., 2001b).

3. CO$_2$-enrichment experiment at the Kennedy Space Center, FL, where 16 large (3.5×3.5 m) octagonal open top chambers (OTC) and associated infrastructure for continuous exposure to ambient and elevated $^{13}$CO$_2$ (350 $\mu$L L$^{-1}$ above ambient) operate in a scrub ecosystem (Drake et al., 1999). Litter from two species: Quercus myrtifolia and the N$_2$ fixer Galactia elliottii was collected at this site, from six OTCs (three at ambient $^{13}$CO$_2$ and three at elevated $^{13}$CO$_2$).

Between the end of October and the beginning of November 1998, naturally senesced leaf litter was collected from the ground at each experimental site, air-dried and stored in paper bags until used for the palatability trials. Litter was kept separately for each site, species, $^{13}$CO$_2$ treatment and the experimental plot from which it was collected. At each site ambient and elevated $^{13}$CO$_2$ experimental plots were paired, so that three pairs of experimental plots were created at the Duke and at the Florida sites and two at the Oak Ridge site.

2.2. Isopod feeding trial

For the feeding trial, isopods of the Porcellio sp. were used. On 11 November 1998, over 1000 individuals, ranging in mass from 2 to 30 mg, were collected from a compost heap in a mixed deciduous forest in Edgewater, MD, USA. They were kept in a glass jar, with holes in the lid to aerate the environment, and fed on the same mixed organic material they were living on prior to collection. The day before the incubation started (day 0), they were left on a starvation diet, inside a moistened glass jar, to empty their gut.

For each of the seven litters (i.e. A. rubrum, C. canadensis, P. taeda, L. styraciflua from Duke and Oak Ridge, G. elliottii, Q. myrtifolia), three types of units were established per pair of experimental plots: A, containing 2 g air-dried litter derived from the ambient $^{13}$CO$_2$ plot; E, with 2 g air-dried litter derived from the enriched $^{13}$CO$_2$ plot; AE, containing 1 g of air-dried litter derived from the ambient $^{13}$CO$_2$ plot and 1 g from the paired enriched $^{13}$CO$_2$ plot.

On day 0 (16 November 1998), 2 g of air-dried intact litter, with petioles removed for broadleaves, was enclosed in a 250 ml plastic beaker (Fischer Scientific, USA), and moistened to 200% water content, by addition of 4 ml deionised H$_2$O. Units were as described above, with a total of 80 units being established. For G. elliottii, only 1 g air-dried leaf litter, and 2 mL H$_2$O, were used because of insufficient material. A litter sub-sample from each litter type was oven-dried at 70 °C for 72 h, for correction of air-dried mass. All data reported are expressed on a oven-dry weight basis.

On day 1 (17 November 1998), seven isopods were added to each unit (total isopod fresh weight per unit ranged between 23 and 46 mg) and units incubated in a humidified cabinet in the dark at 18 °C. A sample of a few isopods was weighed and frozen at $-78^\circ$C until freeze-dried and used for isotope analyses. On day 2, at a careful check of each unit, a total of 28 isopods were found dead and replaced with live ones. In one unit, three isopods were dead and replaced. In five units, two isopods were replaced, and in the remaining 15 units only one isopod was replaced per unit. A similar check was made on day 3, and 10 more isopods,
all from different units, were found dead and replaced. In all these units isopods died because they were trapped in a film of water. In the few units where excess water (not absorbed by leaves) was observed it was removed from the beakers.

On day 14, isopods were removed from the units, counted, weighed, and freeze-dried. In several units, only five or six isopods were recovered, probably because isopods feed on each other. In seven complete units (i.e. A, E and AE) it was possible to recover faecal pellets: from one set with P. taeda litter, one with A. rubrum, all three with C. canadensis and two with L. styraciflua; these pellets were collected and freeze-dried. From all units, litter was oven dried at 40 °C and weighed for determination of mass loss. The dried isopods, litter and faecal pellet samples were stored until used for δ13C analyses.

2.3. Microbial feeding trial

For the microbial feeding trial, the litters derived from all sites listed above were used, and incubation units established following the same scheme as for the isopods trial, with one A, one E and one AE unit for each pair of experimental plots, for a total of 60 units. A known amount (ranging from 0.05 to 0.2 g, depending on species) of air-dried leaf litter of each litter type was incubated, on the top of a 1 cm layer of glass-wool, in a 7 mL plastic vessel; litter was moistened to 100% water content by addition of deionised water in the vessels. Vessels were closed with a moist cotton cup to minimize water loss from litter and incubated in the dark at 15 °C.

On five occasions during the 30 days of incubation, the δ13C of the CO2 respired from litter was measured. On the day of sampling, each vessel had CO2 removed from headspace by circulating CO2-free air and then closed airtight. After incubation for 2 h at 15°C, 2 mL air samples were collected from the vessel, by a syringe inserted in the septum of the lid, and injected in a gas-chromatograph coupled with a mass spectrometer (Finnigan MAT 252) for the determination of δ13C-CO2. Following measurements, deionised water was added to each vessel to replace eventual water loss, determined gravimetrically.

2.4. Chemical analyses

All litter samples were analysed for their C and N concentrations in an elemental analyser (Carlo Erba NA 1500).

The C isotopic composition of leaf litter, isopods and faeces was measured on a mass spectrometer coupled with an elemental analyser (Finnigan Delta plus). For litter, a sub-sample from each type was milled and 2 mg weighed in a tin cup for the analyses of δ13C. Similarly, 2 mg of faecal material from each unit were placed in a tin cup and analysed for δ13C. For isopods, two isopods from each unit were placed in a tin cup and analysed for δ13C.

2.5. Data analyses

The mass balance of stable isotope was applied to the data on faecal pellets and isopods to determine isopods’ feeding preferences. In the AE-mixture unit, the fraction of pellets derived from A litter (fA) was calculated as follow:

\[ f_A = (\delta_{AE} - \delta_E)/(\delta_{AE} - \delta_A) \]

with \( \delta_{AE}, \delta_A \) and \( \delta_E \) being, respectively, the δ13C of faecal pellets from the paired AE, A and E unit. As a consequence the fraction derived from E litter was \( f_E = (1-f_A) \).

A similar approach was applied to calculate the fraction of the isopod’s body derived from the litter (fL) on which they were fed

\[ f_L = (\delta_{HI} - \delta_{HF})/(\delta_{HI} - \delta_{HL}) \]

with \( \delta_{HI}, \delta_{HF} \) being the δ13C of isopods prior to and after incubation and \( \delta_{HL} \) the δ13C of litter. In the AE-mixture unit a weighted average of A and E litter δ13C values was used as \( \delta_{HL} \).

Data were processed using SAS system v. 8.1 (SAS inc.) and Origin v. 6. Statistically significant differences among litter species and 14CO2 treatment, for mass loss and fraction of the isopod’s body derived from litter were assessed applying one and two way ANOVA.

Paired t-tests were applied to assess significance differences between δ13C values of litter and those of faecal pellets and respired CO2. Values were paired per unit.

3. Results

3.1. Litter quality and δ13C

The elevated 14CO2 treatment did not affect the N concentration of the litter examined, either significantly or consistently (Table 1). Litter used in this study exhibited a wide range of δ13C of isopods prior to the incubation (Table 1). As expected, litter under enriched 14CO2 had a lower δ13C than the respective litter under ambient 14CO2 for all species and study sites. However, variability among experimental plots of the same species and study site was observed for the δ13C of elevated 14CO2 litter, with the highest change of 7‰ being observed for the P. taeda litter produced in different rings of the FACE facility at Duke (Table 1).

3.2. Isopods δ13C

The C isotope ratios of the isopods prior to the incubation averaged −22.8 ± 0.06‰. After 14 days incubation their C isotope composition had changed towards that of
Values are means with standard errors; n = 3 for Duke and SERC and n = 2 for ORNL.

the litter on which they fed (Table 2), with a mean δ13C of −24.6, −27.7 and −26.6, respectively, for isopods raised on A, E and AE litter. Also, the faecal pellets produced by the isopods during incubation showed a C isotope composition that did not statistically differ (paired t-test) from that of the litter on which the isopods were fed (Fig. 1). Using Eq. (1), the relative composition of faecal pellets in the mixture AE unit was assessed. Because of the limited number of units sampled, a thorough statistical analyses could not be performed. However, in the faecal pellets retrieved from the A. rubrum, P. taeda and L. styraciflua (from Oak Ridge) mixture units, 79, 81 and 85%, respectively, of the C was derived from the A litter, whereas in those retrieved from the C. canadensis units only 31 ± 8.5% of their C was derived from the A litter.

3.3. Litter palatability

The fractions of C in the isopods derived from ambient, elevated or mixed AE litter (fC), calculated accordingly to Eq. (2), are reported in Fig. 2. Independently of species, litter raised under ambient or elevated 13CO2 appeared to be equally palatable to isopods, and no significant treatment difference was observed for any species, except for the Q. myrtifolia E litter which was eaten more than the respective A litter (Fig. 2). Independently of the litter source, at the end of the experiment a significant percentage of the isopod body was composed of C derived from the litter (Fig. 3a). Isopod C assimilation varied among the different litter species and was significantly correlated with total C loss from the litter during the incubation, although it could explain only a minor fraction of the C loss (Fig. 3b). During the 2 weeks of incubation, litter mass loss values ranged from 2 to 14% depending on the species (data not shown). Statistically significant differences were found among different species, but no effects of elevated 13CO2 were observed, and for all the species, E litter decomposed at a similar rate to A litter. No difference between 13CO2 treatments was observed for the AE-mixture units.

Table 2
Carbon isotope ratios (δ13C) of adult isopods of the Porcellio sp. fed on leaf litter grown under ambient (A) and elevated (E) atmospheric CO2 or on a mixture of the two litters (AE) for 14 days

<table>
<thead>
<tr>
<th>Site</th>
<th>Species</th>
<th>δ13C (%ε)</th>
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<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Duke FACE</td>
<td>Pinus taeda</td>
<td>−23.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Cercis canadensis</td>
<td>−26.7 ± 0.5</td>
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<tr>
<td></td>
<td>Acer rubrum</td>
<td>−25.4 ± 0.3</td>
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<tr>
<td></td>
<td>Liquidambar styraciflua</td>
<td>−25.3 ± 0.1</td>
</tr>
<tr>
<td>ORNL-FACE</td>
<td>Liquidambar styraciflua</td>
<td>−24.4 ± 0.2</td>
</tr>
<tr>
<td>SERC-OTC</td>
<td>Galactia elliottii</td>
<td>−23.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Quercus myrtifolia</td>
<td>−22.9 ± 0.1</td>
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Values are mean with standard errors (n = 3). Before the incubation, the δ13C of the isopods was −22.8 ± 0.06‰.
litter decomposed over a 30-day period and the carbon isotope ratios of the CO₂ released from this respiratory activity.

On average, 16.3% of the litter was decomposed during the 30-day experimental period. Litter produced under either ambient- or elevated-atCO₂ conditions decomposed at the same rate in our experiment (Mann–Whitney $U = 15.0, P = 0.69$). Additionally, there were no significant changes in the mean C isotope ratios of litter between the beginning and end of the experimental period (Mann–Whitney $U' = 906.50, P = 0.83$).

A significant linear relationship between the C isotope ratio of the CO₂ released by microbial decomposition of the litter and the substrate isotopic composition was observed in all units (Fig. 4). There was a tight relationship between the isotopic composition of CO₂ evolved during microbial decomposition and that of the substrate. Yet, the regressed slope of each of the relationships was less than unity, implying the potential for differential fractionation during decomposition (Table 3). However, only the observations on day 30 had a slope that was significantly less than 1 ($P < 0.05$).

We then calculated the potential for C isotope discrimination during microbial decomposition among individual treatments (A, E, AE) on different days following litter incubation. The calculated C isotope discrimination was always significantly greater than 0 (Table 4), implying some C isotope fractionation during decomposition. There was a tendency for microbial decomposition of litter produced under elevated CO₂ to fractionate more than for litter produced under ambient conditions and for this fractionation to increase over time. This tendency is consistent with the slope data (Table 3), which yielded slopes apparently less than 1 and with the shallowest slope on day 30. However, because of the limited sample sizes and large standard deviations, none of the calculated C isotope discrimination values were significantly different from each other at the 0.05 level (although the day 30 ambient atCO₂ versus elevated atCO₂ comparison gave a $t$ value of 2.68, $P = 0.055, df = 19$).

The mass balance of stable isotopes was applied to calculate the relative contribution of the CO₂ derived from A and E litters to the CO₂ respired from the AE units. Results were somewhat different among individual units and a clear trend did not appear, suggesting no preference for any of the treatments.
for ambient- or elevated-atCO₂ litter by the microbes (data not shown).

4. Discussion

Carbon dioxide enrichment experiments are a useful source of ¹³C-labelled material, thus opening a broad range of applications for studies on the effects of elevated CO₂ on C cycling (Pataki et al., 2003), which will certainly contribute to the advancement of the understanding of future changes in C cycling in terrestrial ecosystems.

In the current experiment, δ¹³C values of P. taeda needles generated under elevated ¹⁴C- CO₂ in different experimental plots of the same FACE experiment, showed up to a 7‰ variation between plots. Smaller between-plot differences were measured for the other litter species, although these were greater than the variations in δ¹³C measured in the corresponding ambient litter (Table 1). Differences were also observed between litter of different species derived from the same experimental plot. In pine litter, this variation may have been caused by the fact that needle litter is derived from needles generated over a range of years. Thus pine litter may have carried the δ¹³C signal of the atmospheric CO₂ previous to the treatment. However, the variation may also be related to differential distribution of theatCO₂ enrichment within the plot and along canopy height. In this respect, plant δ¹³C is shown to be a useful tool to assess homogeneity in the CO₂ enrichment of experimental plots.

This study confirmed that stable C isotopes enable C flow to be traced along the decomposer food chain, from the litter to the consumers’ biomass and their bioproducts (i.e. faeces and CO₂), thereby opening a broad range of applications in

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<th>Table 3</th>
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<td>A comparison of the slope (±standard deviation), intercepts, R² value, and probabilities of significance for the relationships between the C isotope ratio of CO₂ evolved by microbial decomposition and the isotope ratio of litter produced under ambient CO₂, elevated CO₂, and a 50:50 mixture treatment</td>
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<tr>
<td>Days following incubation initiation</td>
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<td>Day 1</td>
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<td>Day 30</td>
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<td>The calculated C isotope fractionation associated with microbial decomposition of litter over time for treatments involving litters produced under ambient (A) or elevated (E) CO₂, and a 50:50 mixture (AE)</td>
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<tr>
<td>Treatment</td>
</tr>
<tr>
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for ambient- or elevated-¹⁴CO₂ litter by the microbes (data not shown).
soil ecology. The $^{13}$C label was, in fact, clearly transferred from the litter source to the isopods' bodies (Fig. 2) and faeces (Fig. 1), even when litter and isopods' biomass differed only slightly in their original $^{13}$C values (i.e. when ambient litter was used). We highlight here possible improvements for further applications of this methodology, which we highly encourage. First, since the greater the initial difference in $^{13}$C between source material and feeder the higher the chance of making an accurate estimate of C flow, we suggest raising the feeders on a litter source different in $^{13}$C, as for example on C4 litter (i.e. with a $^{13}$C of around $-14\%$), prior to using them in the feeding trial. In addition, since the insect exoskeleton has a lower turnover rate than the inner body parts, it may carry the $^{13}$C signal of the original diet, diluting the signal from the newly assimilated C. To overcome this problem, it may be advisable to analyse only the inner body parts (i.e. gut) for $^{13}$C. Faeces were shown to be a sensitive material to use when animal feeding is assessed by means of isotopes, and great care should be taken in collecting and analysing them. Finally, isopods were confirmed to be omnivorous (Dickinson and Pugh, 1974), with cannibalistic behaviour, feeding on other isopods. Thus, individual organisms should be used in incubations for feeding trials on plant litter material.

Despite these problems, which limited our ability to quantify preferential feeding in the mixed units, we could accurately determine the amount of C in the isopods' body derived from the litter on which they fed (Fig. 2). We also showed that this was positively correlated to litter mass loss, indicating preferential degradation of substrates enriched in $^{13}$C as compared to the overall litter. Unfortunately, we did not identify the $^{13}$C of specific compounds nor their relative concentrations at the beginning and at the end of the incubation. However, it is well documented that litter C-compounds differ in their isotopic composition, with more negative values being associated with lignin and higher values with more decomposable fractions such as non-acid detergent fibre and cellulose (Fernandez et al., 2003; Schweizer et al., 1999).

With this study, we further demonstrated that the use of isotopically labelled litter can strongly contribute to the advancement of the understanding of litter decomposition and of the quantification of C fluxes in the process.

Acknowledgements

This work was supported by a Smithsonian Visiting Research Scholarship. We are grateful to Rich Norby and William Schlesinger for access to FACE facilities and to Adrien Finzi and Roser Matamala for their help during litter collection.

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