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Stable isotope variation during fungal colonisation of leaf detritus in aquatic environments

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Measuring C and N stable isotopes at natural abundance can provide information on the role of fungi associated with litter decay in the nutrient cycle of freshwater ecosystems. However, uncertainty regarding isotopic fractionation by decomposer fungi during uptake or metabolic turnover is a serious limitation, weakening the description of taxon-specific ecological differences in nutrient transfer in aquatic detritus-based systems. We performed two laboratory experiments to assess C and N isotopic changes during leaf litter colonization by: (1) mixed fungal communities on three different leaf litter species, and (2) four different fungal strains growing on the same leaf litter. Our approach served to decouple the isotopic effects of different fungal taxa from those arising from structural changes occurring in leaf litter during decomposition. N isotopic changes were directly related to fungal biomass accrual on litter, whereas carbon isotopic changes were mainly dependent on the remaining lignin fraction. Isotopic mixing model equations enabled us to calculate strain-specific isotopic fractionation, indicating that fungi were enriched in ^{15}N by 3.6–5.9‰ with respect to bulk N litter.

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Introduction

Fungi play a fundamental role in N and C cycles in ecosystems. By promoting nutrient circulation between different ecosystem compartments, they are important contributors to overall ecosystem functioning and productivity. Fungi associated with litter-decay in lotic systems represent a key step in the transition from primary production to detritus-based systems (Moore et al., 2004), playing a critical role in nutrient transfer between terrestrial and freshwater environments. Fungal biomass and growth depend on the availability and quality of substrates, as well as on water chemistry and temperature (Sabetta et al., 2000; Koide and Malcom, 2009; Boberg et al., 2011; Calizza et al., 2012; Sterflingler et al., 2012; Jabiol et al., 2013). Decomposer fungi are predominant contributors to leaf litter-based food-webs, representing 70–99 % of microbial biomass on decomposing leaf material in freshwaters (Kuehn et al., 2000; Hieber and Gessner, 2002). Fungal colonisation modifies the attractiveness and palatability of detritus for detritivores (Rossi et al., 1983; Suberkropp, 1992; Asplund and Wardle, 2012) and influences, both directly and indirectly, litter decomposition rates and nutrient recycling, depending on species identity and interactions (Bärlocher, 1985; Costantini and Rossi, 2010;

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Cabrini et al., 2013; Calizza et al., 2013a). The physiology and feeding ecology of litter-associated fungi are, thus, key factors to understanding nutrient cycling and ecosystem functioning at the terrestrial-aquatic interface.

A useful technique to assess the role of fungi in C and N dynamics is to measure stable isotopes at natural abundance (Mayor et al., 2009; Hobbie and Högberg, 2012). Due to differences in isotopic composition between various natural compounds, the isotopic signature of fungal biomass can provide information on the substrates used by fungi (Gebauer and Taylor, 1999; Hobbie et al., 2004, 2012). Specifically, the N stable isotope ratio ($^{15}N/^{14}N$, expressed as $\delta^{15}N$) differs between organic and inorganic N sources and between different N forms (Takebayashi et al., 2010; Hobbie et al., 2012), making it possible to clarify internal N transformations and fungal discrimination between different available N pools. In the same way, there are differences in the C stable isotope ratio ($^{13}C/^{12}C$, expressed as $\delta^{13}C$) between different plant litter compounds, and differences can arise following carbon incorporation into different fungal structural compounds (Hobbie, 2005; Hobbie et al., 2012). For instance, cellulose is richer in ^{13}C than lignin (Benner et al., 1987; Ngao and Cotrufo, 2011), and chitin has less ^{13}C than proteins (Hobbie et al., 2012). However, the interpretation of natural N and C isotopic patterns is still limited by a number of considerations. Species-specific differences in isotopic fractionation (i.e. discrimination against one stable isotope during substrate transformation) during uptake or internal reactions within the fungi could, potentially, complicate the use of stable isotopes at natural abundance. Limited knowledge of these differences makes it hard to infer the role of fungi in ecosystem nutrient cycles and to detect taxon-specific ecological differences (Henn and Chapela, 2004; Hobbie and Högberg, 2012).

The majority of studies using stable isotopes in fungal ecology have focused on basidiomycete sporocarps (Griffith, 2004; Hobbie et al., 2001, 2004, 2012; Mayor et al., 2009) and, to a lesser extent, on fungal communities below ground in terrestrial environments (Abraham and Hesse, 2003; Lindahl et al., 2007; Semenina and Tiunov, 2010, España et al., 2011). On the other hand, stable isotope-based studies of nutrient dynamics in freshwaters have focused mainly on bacteria and microalgae (Rysgaard et al., 1993; De Brabandere et al., 2002; McCallister et al., 2004) or addressed the microbial compartment as a whole, with no distinction between fungi and bacteria (Peterson et al., 2001; Hall and Tank, 2003; Webster et al., 2003). Field studies have compared the isotopic signature of fungi and their principal resources, describing the overall isotopic enrichment from substrates to fungi and identifying differences in isotopic patterns between different fungal life forms (i.e. ectomycorrhizal *vs* saprotrophic fungi). Laboratory studies have relied on fungus cultivation on simple growth media, allowing control over nutrient limitation, nutrient forms, growth conditions and the isotopic signature of substrates. Nevertheless, important limitations arise when trying to use laboratory findings to explain observed isotopic patterns in complex natural systems. For instance, N isotopic fractionation is expected to be higher under non-limiting than limiting nutrient availability, higher during assimilation of organic than inorganic N, and higher in liquid than solid media (Hobbie and Högberg, 2012 and literature cited therein).

The impossibility of clearly separating fungal mycelium from decaying plant tissues in freshwater systems limits the direct description of isotopic patterns and fractionation between fungi and their substrates. This makes it hard to discriminate between the direct effect of fungal biomass and the effect of structural changes occurring in litter during decomposition on the isotopic signature of the mycelium-plant litter mix. Such information would help to interpret field observations of isotopic patterns and clarify the crucial role of fungi in the nutrient cycle and nutrient transfer from litter to detritivores feeding on colonised detritus in freshwaters (Rossi, 1985; Costantini and Rossi, 2010; Potapov et al., 2013). To our knowledge, this is the first experiment addressing isotopic changes following fungal colonisation of natural litter in controlled freshwater environments.

To describe isotopic patterns following fungal colonisation of decaying leaf litter, we analysed the mycelial biomass, isotopic signal and relative C and N content of leaf litter during decomposition in freshwater microcosms. We tested the hypothesis that fungus-driven leaf decomposition is accompanied by increased $\delta^{15}N$ ratio in the colonised substratum, seeking to determine whether the observed isotopic changes associated with fungal biomass accumulation on leaf litter can be decoupled from those associated with structural changes occurring in leaves during decomposition. We performed two laboratory microcosm experiments using allochthonous leaf litter and fungi collected from a lake. Specifically, to test: (1) the effect of leaf type three different leaf litter species (alder, beech and reed) were incubated with a natural microbial inoculum; and (2) fungus-specific effects samples of alder litter were individually inoculated with four different fungal strains, seeking to determine potential inter-strain differences in N isotopic fractionation during litter decomposition.

Materials and methods

Experiment 1. Aquarium microcosms with natural microbial inoculum: effect of microbial activity on leaf detritus

Senescent leaves of alder (*Alnus glutinosa*), reed (*Phragmites australis*) and beech (*Fagus sylvatica*) were collected prior to abscission in late autumn around lake Vico (Viterbo, central Italy), as these are the most abundant species among the natural decaying litter. Leaves were air-dried and stored in a dry room until the beginning of the experiments. In late Jan., lake sediments were sampled at 1 m water depth by grab and interstitial water was extracted by filtration. Three aquaria containing 150 l of tap water (sterilised by filtration) and 60 leaves per plant species per aquarium were inoculated with 2 l of the lake sediment interstitial water to start the microbial colonisation of leaves in the aquaria. Water temperature was maintained at 15 $\pm$ 0.5 °C, which corresponded to the lake water temperature at the time of sediment collection. The aquaria were aerated using a continuous-flow air pump supplying filtered air (1 μm). Twenty leaf disks (9 mm Ø) from 5 leaves of each plant species per aquarium were randomly sampled at 7, 21, 35 and 56 d to assess leaf mass loss, ergosterol concentrations (reported as μg of ergosterol per gram of

leaf litter), lignin content (%), C/N values and C and N stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$; see Analysis of samples section). Leaf-disks were sampled from different leaves at each sampling time. Before incubation, leaves were first leached for 36 hr in sterile flasks and then sterilised by autoclaving (at 120 °C for 20 min). Twenty leaf disks were cut from leaves at the time of collection and after leaching, in order to determine initial reference values and the effects of leaching on dry mass, C/N values, lignin content and $\delta^{13}C$ and $\delta^{15}N$ for each plant species. To unambiguously quantify the effect of fungi on the observed results, bacterial growth was inhibited with streptomycin sulphate (Bayer) (25 ml l^{-1}) and Oxytetracycline Hydrochloride in liquid form (Pfizer Limited) (25 ml l^{-1}), which are known to inhibit bacterial growth while not affecting fungal activity (Anderson and Domsch, 1973; Beare et al., 1992). Pre-trial tests showed that this mix reliably inhibited bacterial activity in our microcosms. To monitor unintended fungal effects on leaf isotopic signature, three control aquaria were set up as described above but with no addition of lake sediment interstitial water, and ergosterol concentration, $\delta^{13}C$ and $\delta^{15}N$ were measured after 21 and 56 d.

Experiment 2. Effect of different fungal strains on alder litter

Four fungal strains were isolated from lake Vico littoral detritus using particle plating and dilution techniques (Rossi et al., 1983; Calizza et al., 2013a): *Cladosporium cucumerinum*, *Trichoderma* sp., *Gliocladium* sp. and *Penicillium purpurogenum*. Each strain was grown on liquid agar-malt-extract for 8 d at 15 ± 0.5 °C and then individually inoculated into 250 ml Erlenmeyer flasks containing 140 ml of sterile tap water and 80 leaf disks (9 mm Ø) of alder, previously weighed and sterilised by autoclaving (at 120 °C for 20 min). For each of the four fungal strains, 8 flasks were inoculated by adding 10 ml of a suspension of pure fungal strain. A further 8 flasks containing water and leaf disks were not inoculated and used as controls. To assess mass loss, ergosterol concentrations, lignin content, C/N and $\delta^{13}C$ and $\delta^{15}N$, leaf disks were analysed at the start, after 36 hr of leaching but before autoclaving, and at 3, 7, 14 and 22 d after inoculation, at each sampling occasion collecting all leaf disks from two inoculated flasks per fungal strain and two control flasks. Such procedure limited the number of replicates per sampling time for each fungus treatment, but avoided pseudoreplication, and was preferred in order to test our hypotheses by means of a linear correlative approach. As with the first experiment, bacterial growth within flasks was inhibited by the use of streptomycin sulphate (Bayer) and Oxytetracycline Hydrochloride in liquid form (Pfizer Limited).

Analysis of samples

Leaf mass loss and ash free dry matter as a percentage (AFDM %) were assessed after oven-drying (at 60 °C for 72 hr) and burning (at 550 °C for 6 hr), respectively. Biomass loss was estimated as: $W_t = W_0\, e^{-kt}$ (Olson, 1963), where W_t = leaf mass at time t, W_0 = leaf mass at start, and k = decomposition rate. Lignin content was determined by the $NaClO_2$ method (Rahn et al., 1999) using 0.2 g of leaf disks. Ergosterol was extracted using the reflux in methanol method according to Sabetta et al. (2000) and analysed by HPLC (Waters 996 spectrometer and column reversed-phase μBondapak C18 at 280.5 nm). Ergosterol concentration was converted into fungal biomass, using a conversion factor of 5.5 mg ergosterol g^{-1} fungal dry mass in accordance with Gessner and Chauvet (1993).

The stable isotope signatures and relative C and N content of leaf litter were determined in a Elementar vario-MICRO CUBE analyser (Elementar, Hanau, Germany) coupled with an Isoprime 100 mass spectrometer (Isoprime Limited, Cheadle Hulme, UK), operating as a continuous flow system. Samples were dried and powdered (Post, 2002); for each sample, 0.800 ± 0.001 mg of lyophilised powder was introduced into tin capsules for solids (3.5 × 5 mm). The outputs were standardised with caffeine. All samples were analysed twice and values were averaged. Isotope values are expressed as $\delta^{13}C$ and $\delta^{15}N$ with units of ‰, in accordance with the following equation: $\delta X(‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3$, where X = ^{13}C or ^{15}N, and R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Ponsard and Arditi, 2000; Vander Zanden and Rasmussen, 2001). Reference standards were PeeDee Belemnite carbonate for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$ (Peterson and Fry, 1987).

In the second experiment, the $\delta^{15}N$ of fungal biomass for each fungal strain was calculated in accordance with the following mixing model:

$$\delta^{15}\text{N fungus} = [(\delta^{15}\text{N colonised litter} - \delta^{15}\text{N control})/(\text{fungal biomass per gram of leaf litter})] - (\delta^{15}\text{N control}),$$

where the control is the non-inoculated litter.

N isotopic enrichment of each fungal strain at each sampling occasion was then obtained as: $\delta^{15}N$ fungus − $\delta^{15}N$ control.

Data analysis

As the lignin content of decaying litter was always found to be strongly related to changes in other structural components of litter, i.e. hemi-cellulose, alpha-cellulose, dry weight and ash free dry mass (AFDM) (Fig S1), and as the lignin fraction has been shown to affect the carbon isotopic ratio of decaying leaf material (Benner et al., 1987), we here make reference to changes in lignin content, considering this a good indicator of changes in the structural properties of leaf litter during the experiments. We considered the model with the lowest Akaike's Information Criterion (AIC) (Akaike, 1976, 1981) as the best model of fit for changes in lignin and the other parameters under study. The extensive description of model selection is included in the online supplementary material (Tables S1 and S2).

The three null hypotheses of no relationship between (a) ergosterol concentration (as a proxy for fungal biomass) and $\delta^{15}N$, (b) C/N and $\delta^{15}N$, and (c) lignin content (as a proxy for leaf structural changes) and $\delta^{13}C$, were tested by means of linear regression with n = 18 for single leaf species (3 aquaria × 6

sampling times) and $n = 54$ for entire dataset (18 observations × 3 leaf litter species) in experiment 1, and $n = 12$ for single fungal strains (2 flasks × 6 sampling times) and $n = 60$ for entire dataset (12 observations × 5 treatments) in experiment 2. The paired *t*-test was applied to test for a significant effect of fungi (i.e. the mean effect observed between the four fungal strains with respect to the control) on lignin and isotopic signatures of decaying litter. Two-way ANOVA was applied to test the effect of time and aquaria and their interaction on the % of lignin, $\delta^{13}C$, $\delta^{15}N$ and C/N for each leaf litter species. As time had a significant effect in most cases, two-way ANCOVA, with time as covariate factor, was applied to test the effect of leaf species and aquaria and their interaction. In the cases where time had no significant effect, two-way ANOVA was applied to test these effects. Values in the text and in figures are shown as mean ± standard error.

Results

Experiment 1: effect of leaf type

Alder, beech and reed leaves had different initial biochemical characteristics, which changed differently during decomposition (Table 1). Leaf litter ergosterol concentration in all three leaf species followed a parabolic trend, peaking after 21 d, though fungal growth was very slow during the first week in reed (Fig S2 and Table S1). Leaf mass decreased exponentially in all leaf types during the experiment, litter half-life being 38.5 d in reed, 31.5 d in beech and 28.8 d in alder, corresponding to significantly different decomposition rates (ANCOVA and Homogeneity of Slopes Test, $F = 13.6$ $p < 0.0001$) (Table 1).

All parameters under study differed significantly between leaf litter species and among sampling times, with the only exception of the $\delta^{15}N$ in reed and $\delta^{13}C$ in alder. Neither significant differences between aquaria, nor significant interaction effects (i) between aquaria and time, and (ii) between aquaria and leaf litter species were observed (Table S3 and Table S4). In particular, the starting isotopic signals of leaf species differed (Table 1) and showed species-specific variations during decomposition (Fig 1 and Table S1). Initial $\delta^{13}C$ was highest in reed, followed by alder and beech (Table 1). $\delta^{13}C$ decreased linearly during the course of the experiment in reed and beech, but followed a parabolic trend in alder (Fig 1 and Table S1). Overall, $\delta^{13}C$ was negatively correlated with leaf tissue lignin content ($y = -0.07x - 26.7$, adjusted $r^2 = 0.19$, $n = 54$, $p = 0.004$), which increased during decomposition in all leaf species (Fig S3 and Table S1).

$\delta^{15}N$ values were higher in reed than in the other two leaf species (Table 1), but the difference between initial and final values was not significant and no clear temporal trend was observed (Fig 1 and Table S1). In contrast, $\delta^{15}N$ increased

Table 1 – Biochemical characteristics of leaf species

	P. australis	*F. sylvatica*	*A. glutinosa*
% AFDM at start	83.0 ± 2.3^a	92.0 ± 1.2^b	94.8 ± 0.9^b
% Soluble substance at start	14.2 ± 1.1^a	19.3 ± 5.5^b	25.4 ± 1.7^b
% Lignin at start	18.2 ± 0.8^a	24.7 ± 1.7^b	32.6 ± 2.5^c
% Hemi-cellulose at start	42.6 ± 2.8^a	34.6 ± 7.9^b	22.7 ± 2.1^b
% Alpha-cellulose at start	19.8 ± 2.3	12.5 ± 0.9	13.0 ± 2.9
Olson's K (day^{-1})	0.018	0.022	0.024
C/N at start	18.0 ± 1.0	21.0 ± 0.6	18.6 ± 0.2
C/N at end	14.9 ± 0.1^a	11.9 ± 0.7^b	11.5 ± 1.1^b
$\delta^{15}N$ at start (‰)	3.7 ± 0.6^a	-2.7 ± 0.1^b	-1.1 ± 0.1^c
$\delta^{15}N$ at end (‰)	4.7 ± 0.6^a	-1.2 ± 0.5^b	-0.5 ± 0.2^b
$\delta^{13}C$ at start (‰)	-25.1 ± 0.2^a	-31.3 ± 0.3^b	-29.7 ± 0.4^c
$\delta^{13}C$ at end (‰)	-27.2 ± 0.1^a	-32.5 ± 0.1^b	-29.4 ± 0.3^c

Different superscript letters (a, b, c) indicate significant differences between leaf species (One-way ANOVA and post-hoc comparison $p < 0.05$).

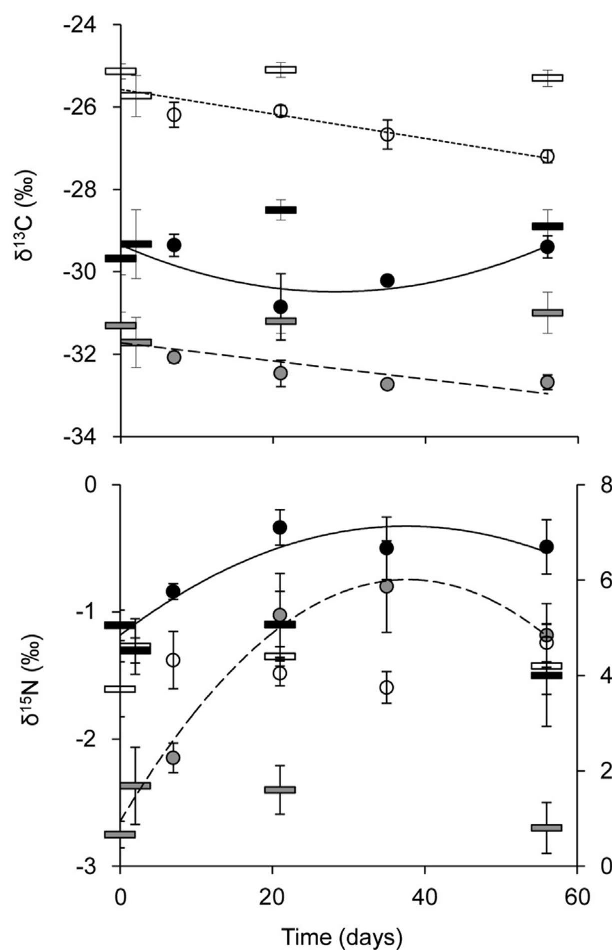

Fig 1 – Changes in $\delta^{13}C$ and $\delta^{15}N$ during litter decomposition by a mixed fungal community colonising three different leaf litter species. Open symbols: *Phragmites australis*; black symbols: *Alnus glutinosa*; grey symbols: *Fagus sylvatica*. $\delta^{15}N$ values of *P. australis* are shown on the right vertical axis. Each circle represents the mean value (±s.e.) between three inoculated aquaria. Rectangles indicate mean values (±s.e.) of non-inoculated leaf litter at start (0 d) and after leaching (set on 1.5 d), and at 21 d and 56 d in control aquaria. Best model of fit is shown for each parameter and each leaf species (see Table S1 for model details). Models do not refer to control aquaria.

during decomposition in beech and alder (Fig 1 and Table S1), the fastest increase being between 7 and 21 d. $\delta^{15}N$ was positively correlated with ergosterol concentration in both beech and alder, but not in reed (Table 2). In the same way, $\delta^{15}N$ was negatively correlated with C/N in alder and beech, but not in reed (Fig 2). In turn, C/N values were negatively correlated with ergosterol concentration for all leaf species (Table 2). C/N variation patterns are shown in Fig S3 and Table S1.

Ergosterol in the leaves was not detected at 21 d and was negligible at 56 d in control aquaria. $\delta^{13}C$ and $\delta^{15}N$ of leaf litter after 21 and 56 d were not significantly different from the values observed after the leaching (Fig 1; one-way ANOVA, $p > 0.05$ in all cases).

Experiment 2: effect of single fungal strains on alder leaf disks

Ergosterol concentration in alder leaf disks increased during decomposition following a parabolic pattern with all fungus strains except *C. cucumerium*, with which it increased linearly (Fig S2 and Table S2). Ergosterol was detected later and at lower values with *Trichoderma* and *Penicillium* than with *Gliocladium* and *Cladosporium* (Fig S2), and was absent in control treatments. The decomposition rate (k) ranged from 0.006 d^{-1} in the presence of *P. purpurogenum* to 0.013 d^{-1} in the presence of *C. cucumerinum* (Table 3), and was positively correlated with mean ergosterol concentration in leaf disks during decomposition ($y = 0.4*10^{-4}x - 1.4*10^{-3}$ adjusted $r^2 = 0.89$, $n = 5$, $p = 0.01$).

On average, the $\delta^{13}C$ of leaf disks in the presence of fungi was significantly lower than the control (Fig 3; paired t-test $t = 3.5$, $n = 4$, $p = 0.04$) and did not vary significantly with time after leaching. By contrast, the $\delta^{13}C$ of leaf disks was negatively correlated with lignin content ($y = -0.08x - 26.79$, adjusted $r^2 = 0.35$, $n = 60$, $p < 0.0001$), which varied in the presence of *Cladosporium* and *Gliocladium* but not with *Penicillium* and *Trichoderma* (Table S2 and Fig S3). Overall, lignin content in leaf disks was positively correlated with ergosterol concentration ($y = 0.01x + 32.95$, adjusted $r^2 = 0.28$, $n = 60$, $p < 0.001$). On average, lignin content across sampling times was higher than the control (paired *t*-test $t = 3.7$, $n = 4$, $p = 0.035$).

$\delta^{15}N$ varied during decomposition following a parabolic trend with all fungus strains except *C. cucumerium*, with which it increased linearly during the experiment (Fig 3 and Table S2). $\delta^{15}N$ increased during the first week even in the absence of fungi, but mean values across sampling times were higher with than without fungi (paired *t*-test $t = 4.8$, $n = 4$, $p = 0.016$).

Specifically, the $\delta^{15}N$ values of leaf disks were positively correlated with ergosterol concentrations with all fungus strains except *P. purpurogenum* (Table 3). In addition, the magnitude of change in the $\delta^{15}N$ of leaf disks during decomposition (i.e. final $\delta^{15}N$ − initial $\delta^{15}N$) was directly correlated with mean ergosterol concentration over the 22 d of the experiment ($y = 1668.1x - 1319.2$, adjusted $r^2 = 0.83$, $n = 5$, $p = 0.02$) (Table 3). As observed in experiment 1, the $\delta^{15}N$ of colonised leaf disks was negatively correlated with C/N values for all fungus strains (Fig 2). In turn, C/N during decomposition was negatively correlated with ergosterol concentration ($y = -0.0025x + 14.74$, adjusted $r^2 = 0.18$, $n = 60$, $p = 0.002$). C/N variation patterns are shown in Fig S3 and Table S2.

The application of mixing model equations to the $\delta^{15}N$ values of colonised leaf disks compared with control disks allowed the computation of the $\delta^{15}N$ of fungal biomass for each fungal strain (Fig 3). $\delta^{15}N$ varied from a minimum of 2.3 ± 0.5‰ with *Gliocadium* sp. to a maximum of 4.6 ± 0.1‰ with *Trichoderma* sp., implying an isotopic ^{15}N enrichment in fungi with respect to non-colonised decaying leaf litter of 3.6 ± 0.5‰ and 6.0 ± 0.1‰, respectively.

Table 2 – Relationships between ergosterol concentration ($\mu g\ g^{-1}$) and (i) $\delta^{15}N$ (‰) and (ii) C/N of leaf litter during decomposition

	Model	d.f.	Adjusted r^2	p value
(i)[Ergosterol](x) *vs* $\delta^{15}N$ (y)				
P. australis	$y = -0.0003x + 4.40$	16	0.06	n.s.
F. sylvatica	$y = 0.0011x - 1.05$	16	0.38	**
A. glutinosa	$y = 0.0035x - 2.36$	16	0.41	**
(ii)[Ergosterol](x) *vs* C/N (y)				
P. australis	$y = -0.0043x + 18.04$	16	0.27	*
F. sylvatica	$y = -0.0155x + 19.51$	16	0.39	**
A. glutinosa	$y = -0.0141x + 16.41$	16	0.47	**

n.s.: $p > 0.05$,*: $p < 0.05$,**: $p < 0.01$.

Discussion

During colonisation and decomposition, the $\delta^{15}N$ varied in both alder and beech leaves colonised by a mixed fungal community (experiment 1) and in alder leaf disks colonised by single fungal strains (experiment 2). The pattern of change was consistent with that of fungal biomass accrual on decaying litter and, given the observed patterns, could not be explained by structural changes occurring in litter during decomposition. In the first experiment, the steepest increase in $\delta^{15}N$ was reported between 7 and 21 d, accompanied by a peak in fungal biomass. Leaf ergosterol concentration was significantly correlated with $\delta^{15}N$ in alder and beech, but not in reed, where $\delta^{15}N$ did not vary significantly during decomposition. We reported higher $\delta^{15}N$ values at intermediate decomposition times, while structural changes in leaves progressed continuously, as indicated by the pattern of lignin content in the remaining leaf material.

The initially delayed but ultimately greater fungal accrual on reed with lake microbial inoculum, which was not coupled with a high decomposition rate or an increase in $\delta^{15}N$, could be explained by preferential nutrient uptake from water, which can represent a complementary nutrient source for fungal growth on decomposing leaves (Suberkropp, 1998; Tank and Webster, 1998; Gulis et al., 2008). Indeed, dissolved inorganic N compounds potentially accumulating in water following leaching, leaf decomposition and fungal metabolic turnover are expected to be characterized by lower isotopic values compared to organic N; moreover, the assimilation of simple N sources can lead to lower or null isotopic fractionation compared to more complex organic N forms (Henn and Chapela, 2004; Takebayashi et al., 2010; Hobbie and Högberg, 2012).

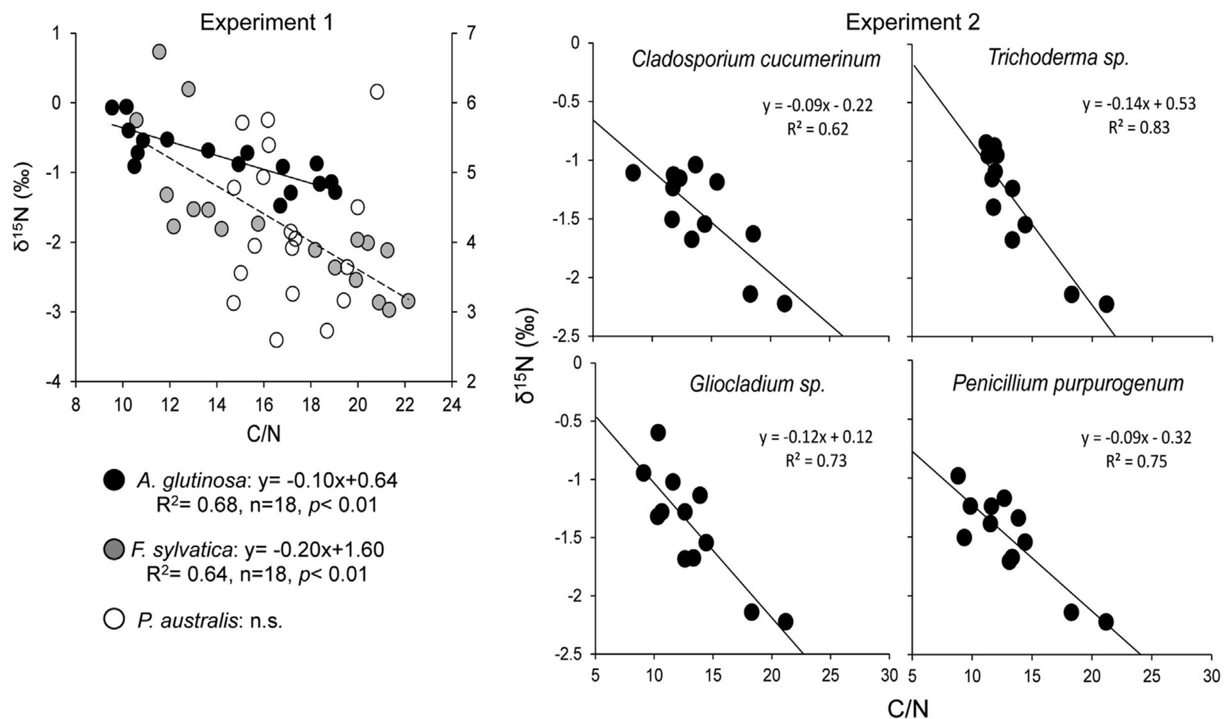

Fig 2 – Correlation between C/N and $\delta^{15}N$ in decomposing litter. Left panel refers to Experiment 1: a mixed fungal community colonising three different leaf litter species. $\delta^{15}N$ of *Phragmites australis* shown on right vertical axis and linear regressions shown under the graph (n.s. = not significant correlation, $p > 0.05$). Right panels refer to Experiment 2: four different fungal strains colonising alder leaf disks; linear regressions, $n = 12$ and $p < 0.01$ for all fungal strains.

As for the whole leaves colonised by a mixed fungal community, the $\delta^{15}N$ of alder leaf disks increased with ergosterol concentration during decomposition, and differences between leaf disks across sampling times were explained by fungal biomass accrual. Specifically, with *Trichoderma* sp. and *Gliocladium* sp., maximum $\delta^{15}N$ occurred after 14 and 22 d, respectively, corresponding to peak fungal colonisation of leaf disks. Consistent with these results, in both experiments the relative increase in %N during fungal colonisation of litter was explained by fungal biomass accrual and was associated with an increase in $\delta^{15}N$, with the sole exception of reed. Considering these results, the relative increase in %N during decomposition may be ascribed to the accumulation of ^{15}N-enriched fungal proteins on the decomposing substratum (Gebauer and Taylor, 1999; Lindahl et al., 2007; Semenina and Tiunov, 2010; Hobbie et al., 2012).

Relative ^{15}N enrichment during leaf material processing has been hypothesised to depend on heterotrophic microbial metabolism and activity, as well as leaching of labile N from leaves (Nadelhoffer and Fry, 1994; Adams and Grierson, 2001; Kramer et al., 2003; Bragazza et al., 2010). In the first experiment, leaching produced no appreciable changes in the $\delta^{15}N$

Table 3 – (i) Decomposition and biochemical parameters of leaf disks colonised by different fungal strains. Control: non-colonised leaf disks. K: leaf litter decay constant; mean (±s.e.) ergosterol concentration as recorded on leaf disks between 3 d and 22 d; $\Delta\delta^{15}N$: final (22 d) – initial (0 d) $\delta^{15}N$; ΔC/N: final (22 d) – initial (0 d) C/N. (ii) Relationship between ergosterol concentration ($\mu g\ g^{-1}$) and $\delta^{15}N$ (‰) of inoculated leaf disks during decomposition

(i) Treatment	K (day^{-1})	[Ergosterol] ($\mu g\ g^{-1}$)	$\Delta\delta^{15}N$ (‰)	ΔC/N
Cladosporium cucumerinum	0.0135	739.8 ± 137.6	1.03	−8.56
Trichoderma sp.	0.0126	374.1 ± 102.3	1.06	−7.95
Gliocladium sp.	0.0324	1 254.4 ± 222.7	1.24	−9.24
Penicillium purpurogenum	0.0006	465.6 ± 193.5	0.92	−6.47
Control	0.0009	0.0 ± 0.0	0.76	−5.74
(ii) [Ergosterol](x) *vs* $\delta^{15}N$ (y)	**Model**	**d.f.**	**Adjusted r^2**	***p* value**
Cladosporium cucumerinum	$y = 0.0005x - 1.70$	10	0.58	*
Trichoderma sp.	$y = 0.0011x - 1.60$	10	0.62	*
Gliocladium sp.	$y = 0.0004x - 1.75$	10	0.66	**
Penicillium purpurogenum	$y = 0.0004x - 1.63$	10	0.38	n.s.

n.s.: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

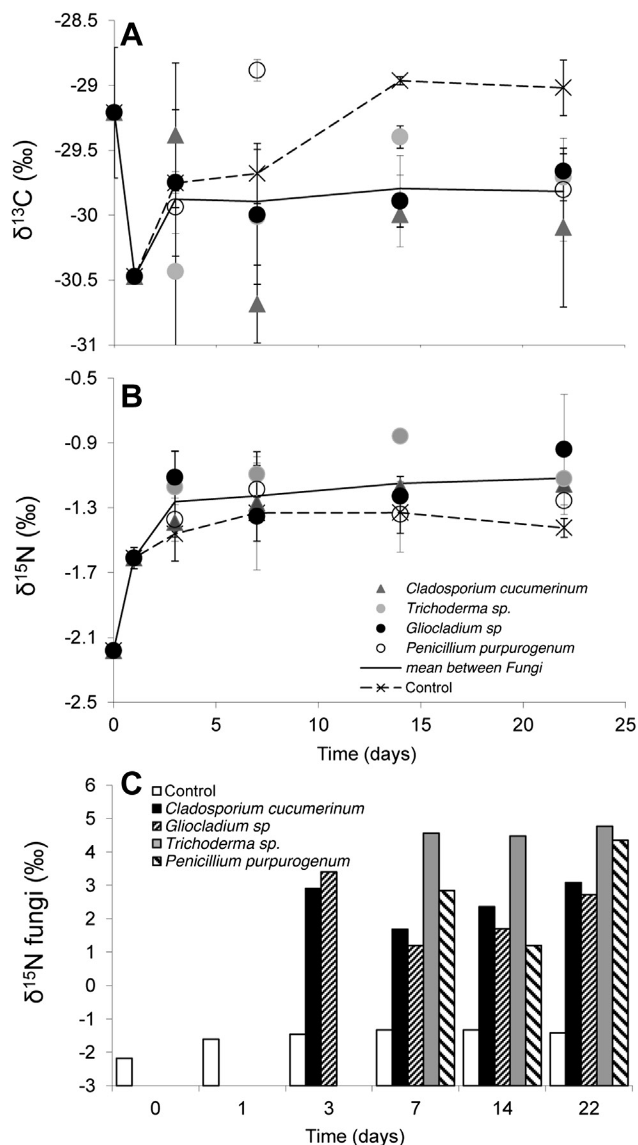

Fig 3 – (A, B) Effect of four different fungal strains on $\delta^{13}C$ and $\delta^{15}N$ values of decomposing alder leaf disks. For the model and statistical significance of the variation for each fungal strain see Table S2. (C) $\delta^{15}N$ of each fungal strain as calculated by linear mixing models, based on $\delta^{15}N$ of colonised leaf disks, fungal biomass per gram of leaf disk (obtained from ergosterol concentration) and $\delta^{15}N$ of non-inoculated (control) leaf disks.

of whole leaves. Similarly, in the second experiment, $\delta^{15}N$ increased by only 0.5‰ in leaf disks during leaching, but increased in parallel with ergosterol accumulation after leaching in all treatments except the control. Thus, further potential leaching occurring after the 36 hr of the initial controlled leaching step should not be considered relevant to the observed $\delta^{15}N$ variations in decomposing litter.

Interestingly, the calculated N isotopic signature of fungal biomass ($\delta^{15}N$) varied from 2.3‰ for *Gliocadium* sp. to 4.6‰ for *Trichoderma* sp. Given the lower $\delta^{15}N$ value of sterile litter, this implies a preferential ^{15}N accumulation in fungal biomass. This can be due to (i) preferential assimilation by fungi of certain leaf compounds that are relatively rich in ^{15}N, and/or (ii) preferential loss from fungi of ^{15}N-depleted N during metabolic turnover. Hypothesis (i) matches the significant relationship between $\delta^{15}N$ and both fungal biomass on leaf litter and the rate of leaf mass loss, implying higher isotopic fractionation was coupled with higher N uptake by fungi from litter. On the other hand, ^{15}N-depleted N loss (point ii) during transamination reactions carried out by fungi can also represent a preferential pathway for relative ^{15}N enrichment (Hobbie et al., 1998, 1999), leading to greater $\delta^{15}N$ for fungal biomass than bulk litter N. Together with strain-specific differences in N uptake by fungi, it can help to explain the differences in $\delta^{15}N$ and isotopic fractionation between the fungal strains. Furthermore, the increase in $\delta^{15}N$ of the fungus-colonised detritus in both experiments implies that fungi lost ^{14}N during metabolic turnover to varying degrees, depending on biomass accrual rate and strain-specific physiological differences.

The relative enrichment in ^{15}N in fungal biomass calculated with mixing model equations is consistent with irreversible reactions dominated by a kinetic isotopic effect (i.e. isotopic fractionation due to a difference in reaction speed between lighter and heavier isotopes) occurring in open, non-nutrient limited systems (Hobbie et al., 2004; Hobbie and Högberg, 2012). The fractionation values fall within the range reported for fungi growing on complex organic substrata (Hobbie et al., 2012; Hobbie and Högberg, 2012), but contrast with other studies performed in terrestrial ecosystems reporting limited N fractionation by fungi (Lindahl et al., 2007; Potapov et al., 2013). Since all fungi were growing on the same leaf substratum under controlled conditions, our results suggest that taxon-specific physiological traits can influence natural N isotopic patterns in freshwater detritus systems, as already observed for terrestrial ecosystems (Hobbie et al., 2004; Semenina and Tiunov, 2010; Potapov et al., 2013). Thus, the quantification of strain-specific conversion factors between ergosterol concentration and fungal biomass will help to improve our ability to quantify reliably the isotopic fractionation by individual fungal species.

In both experiments, lignin content explained the changes in the decaying litter $\delta^{13}C$, which, considering the different pattern in change of lignin content and ergosterol concentration, was driven by structural changes rather than microbial biomass accrual *per se* (Benner et al., 1987; Wedin et al., 1995; Ngao and Cotrufo, 2011). Lignin is generally depleted (between 2 and 6 ‰) in ^{13}C with respect to other leaf compounds with higher decomposability, such as cellulose (Benner et al., 1987; Ngao and Cotrufo, 2011), influencing the $\delta^{13}C$ value of decomposing leaf litter. A study by Semenina and Tiunov (2010), based on different species growing on simple culture media, including *Cladosporium* and *Trichoderma* spp., reported no or small carbon isotopic fractionation by fungi. On the other hand, a recent laboratory study by Potapov et al., (2013) reported substantial isotopic ^{13}C enrichment in a three-trophic level terrestrial detritus system (i.e. natural litter – decomposer microfungi – fungivorous collembola), and the observed isotopic enrichment was ascribed to decomposer fungi. However, the impossibility to completely separate fungal mycelium from the plant litter substratum limits the direct analysis of isotopic patterns and fractionation between fungi and their substrates, and thus to disentangle effects of

lignin depletion and fungal enrichment on the C isotopic signature of decaying litter.

Conclusions

In our experiments bacterial growth was inhibited, but considering that (i) fungal biomass represents up to 95–99 % of microbial biomass on decomposing leaf material (Hieber and Gessner, 2002), and (ii) changes in both N content and isotopic ratios of colonised litter primarily depended on fungal biomass, our results probably parallel changes in naturally colonised decomposing litter in freshwaters. Inoculation with a mixed fungal community and the concomitant use of a mixture of leaf species in our first experiment enhance the similarity to field conditions, where both fungal and leaf litter diversity can significantly influence the activity of decomposer fungi and litter breakdown (Costantini and Rossi, 2010). Knowledge of variation in isotopic fractionation among decomposer microfungi improves our comprehension of both stable isotope patterns in natural systems and the role of specific taxa in nutrient circulation in freshwaters. Quantification of differences in isotopic fractionation by different fungal taxa can also improve the description of trophic pathways in detritus-based systems, as recently suggested for fungivorous microarthropods in terrestrial environments (Potapov et al., 2013). Indeed, differences in δ^{15}N between *Trichoderma* sp. and *Gliocladium* sp. calculated in our second experiment averaged 2.4‰, which is close to the mean isotopic fractionation value expected for invertebrates feeding on decomposing leaf material (McCutchan et al., 2003). Thus, if the potential fungus-mediated effect on the isotopic baseline in freshwater detritus systems is neglected, specialist fungivorous invertebrates could be assigned to the wrong trophic position. On the other hand, for those detritivores unable to discriminate between leaf tissue and microbial biomass during ingestion, consideration of the effects of fungal isotopic fractionation can help to determine litter mixture composition and the detritivores' actual assimilation of one food source rather than another (i.e. detritus *vs* fungi), starting from appropriate isotope signature-based assumptions (Scheu, 2002; Rossi et al., 2010; France, 2011; Calizza et al., 2013b).

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2014.05.008.

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