

Nitrogen transfer in litter mixture enhances decomposition rate, temperature sensitivity, and C quality changes

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Abstract

Background and aims Litter decomposition is a critical process in terrestrial ecosystems and, since in natural conditions plant litter occurs in mixtures, understanding the interactive effects of mixed litter is of great ecological relevance. In this context, we test the hypothesis that N transfer between high quality litter to N-poor substrates are at the base of synergistic interactions, positively affecting litter decay rate, temperature sensitivity, and changes of organic C quality.

Methods We carried out a manipulative experiment using four organic substrates, encompassing a wide range of biochemical quality (*Hedera helix* and *Quercus ilex* leaf litter, cellulose strips and woody sticks), each decomposing either separately or in matched pair mixtures for 360 days. Organic substrates were characterized for mass loss, C and N content and by ^{13}C CPMAS NMR to assess biochemical quality changes.

Results Litter response to mixing was related to the biochemical quality of the components in the mixture: additive when substrates with similarly high (*H. helix* and *Q. ilex*) or low (cellulose and wood) N content were paired, but synergistic when substrates with contrasting N content were associated (either of the two leaf litters with either cellulose or wood). Overall, no antagonist effects were observed in this experiment. Interestingly, decomposition of cellulose and wood showed a higher temperature sensitivity, compared to monospecific substrates, when paired with N rich materials. Significant N transfer was found from N rich litter to N poor substrates and ^{13}C CPMAS NMR showed rapid changes of C quality of cellulose and wood sticks only when paired with N rich litter.

Conclusions Our findings support the hypothesis that mixing litters of different quality, with quality expressed in terms of C/N ratio and N content, increases decomposition rate and temperature sensitivity of the lower quality substrates.

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Introduction

Litter decomposition is a critical process in terrestrial ecosystems, controlling carbon and nutrient cycling and, as a consequence, primary productivity. Decades of studies confirmed that temperature and water availability are

the most important ecological factors affecting decomposition at global and regional levels (Aerts 1997), with biochemical litter quality becoming a relevant factor at a local scale (Bonanomi et al. 2013). Most early decomposition studies used leaf litter from single plant species. However, since in natural conditions plant litter generally occurs in mixtures, in the last decade an increasing research effort was devoted to clarify the decomposition dynamics in litter mixture (Hättenschwiler et al. 2005).

Litter mixtures can decompose as fast as the average of the single components thus showing additive behaviour. In other cases mass loss rate can deviate from the average of its separate components and such non-additive effects may produce either synergistic (faster mass loss) or antagonistic (slower mass loss) interactions. Unfortunately, non-additive interactions of decomposing litter mixtures seems to occur idiosyncratically, being rarely predictable from single-species dynamics. Two reviews concerning mixed litter reported that synergistic effects are slightly more common compared to antagonistic and additive ones, both in terrestrial (Gartner and Cardon 2004) and in aquatic ecosystems (Lecerf et al. 2011), but the underlying causal factors are still unclear. Some authors suggest that non-additive interaction is expected to occur more frequently when mixtures include litter with dissimilar biochemical quality (Wardle et al. 1997). In this regards, however, available empirical evidence is contradictory (Hoorens et al. 2003; Wardle et al. 2006; Bonanomi et al. 2010). Interestingly, Chapman and Koch (2007) reported that litter mixtures of functionally similar conifer species decomposed faster than expected from rates of the individual species alone, whereas more functionally diverse mixtures did not show synergistic effects.

Several mechanisms can explain non-additive, synergistic interactions: i. improved microclimatic conditions since litter materials with higher water retention capacity, providing a water buffering effect, may enhance the decay of fast de-hydrating plant residues by favouring moisture persistence in the litter layer (Wardle et al. 2003); ii. creation, in litter mixture, of more structured habitats that may enhance microbial diversity and functionality (Hättenschwiler et al. 2005); iii. a net transfer of nutrients among different litter materials (Schimel and Hättenschwiler 2007). Nitrogen (N) can be transferred passively, through leaching and diffusion, or actively, by fungal mycelia networking (Lummer et al. 2012). In particular, N transfer becomes ecologically relevant during the early phases of litter

decomposition, in conditions of C/N ratio of the decomposing substrate above a critical threshold of ~30–35, when microbial N starvation is commonly observed (Taylor et al. 1989). In such conditions, decomposer microbes could get extra N from external sources (e.g. the underlying soil) and transfer it into the N-poor substrate to meet their nutritional requirements (Berglund and Ågren 2012). Accordingly, non-additive synergistic interactions are expected to occur when high quality litters (low C/N ratio and high N content) are mixed with N poor substrates, as a consequence of a net N transfer between the two materials. However, this simplistic model does not fully capture the complexity of N related effects on the decomposition process. For instance, a number of studies investigated the effects of N addition on litter decomposition rate, reporting contradictory evidence. In some cases a faster decomposition after N fertilization is observed (e.g. Cornwell et al. 2008), but examples of non significant or even negative responses are common (Berg and McLaugherty 2008). Moreover, a recent meta-analysis (Knorr et al. 2005) reported that, generally, N addition from external sources enhances mass loss of high quality litter, but reduces decay rate of low quality, lignin rich substrates. Such depressive effect has been related to different processes, including the inhibition of oxidative enzymes involved in lignin degradation (Carreiro et al. 2000), the formation of recalcitrant compounds as a result of the interactions between inorganic N and polyphenols (Berg and Matzner 1997), generalised reduced functionality of microbial communities when inorganic N is abundant (Ågren et al. 2001). The complex relationships between litter decomposition dynamics and N availability is furthermore affected by both the type of N source and the biochemical quality of decomposing litter. In a recent study, comparing the effects of substrate N and externally supplied organic and inorganic N on decomposition dynamics, Hobbie et al. (2012) reported contrasting effects. In particular, faster initial decomposition rates were dependent on the level of N availability, but not on its sources and forms. On the other hand, later in decomposition, externally supplied N had a prevalent slowing effect on litter mass loss, contrary to the substrate N.

In the context of litter mixtures decomposition experiments, only few studies have conclusively demonstrated a net N transfer between different starting materials (e.g. Schimel and Hättenschwiler 2007; Lummer et al. 2012). So far, the interactive effects of litter type, in

terms of C biochemical quality, and N transfer dynamics have received even less attention. In this work we have carried out a manipulative, laboratory experiment using four organic substrates, encompassing a wide range of biochemical C quality and N content (*Hedera helix* and *Quercus ilex* leaf litter, cellulose strips and woody sticks), each decomposing either separately or in matched pair mixtures. We tested the hypothesis that a net N transfer from high quality substrates (leaf litter of *H. helix* and, to a lesser extent, *Q. ilex*) to N-poor materials promotes accelerated decomposition rates. A specific hypothesis states that the intensity of synergistic interactions is expected to increase with the difference in N content between the litters in mixture. In particular, a stronger synergistic effect is expected for mixtures containing cellulose strips (N poor and devoid of lignin) compared to woody sticks (N poor, but lignin rich) because, in the latter case, a potential negative interaction between N and lignin may occur (Berg and Matzner 1997; Carreiro et al. 2000; Knorr et al. 2005).

Previous litter mixture studies focused on the effects of mixing on litter mass loss and nutrient dynamics, or on the effects of environmental abiotic and biotic factors on mixture decomposition dynamics (review in Hättenschwiler et al. 2005). So far, no studies investigated if and how monospecific litter and litter mixtures differently affect litter C quality changes during decomposition. In this work, intended as a contribution to fill the gap, undecomposed as well as decomposed materials were characterized by ^{13}C -cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy obtained in solid state. Such method has been applied to characterize organic matter at molecular level and it is useful since it provides a description of the total organic chemical composition of complex matrices, such as plant litter (Kögel-Knabner 2002; Preston et al. 2009). In detail, we tested the idea that mixing N-poor substrates (i.e. cellulose strips and woody sticks) with N-rich litter (i.e. *H. helix* and *Q. ilex* leaves) enhances the general functionality of microbial decomposer community by relaxing N starvation. Under such hypothesis, more rapid changes of C quality are expected in cellulose strips and woody sticks when mixed with leaf litter, as compared to unmixed conditions.

Finally, the relationships between litter decomposition and temperature are fairly well established in the literature (e.g. Meentemeyer 1978; Hobbie 1996), with decomposition rates increasing with increased

temperatures. However, temperature sensitivity of decomposing litter is a debated issue, with contrasting evidences about the relation between sensitivity and substrate recalcitrance (Hartley and Ineson 2008; Giardina and Ryan 2000). Temperature sensitivity of organic substrates can be reduced in presence of environmental constraints such as water and nutrient shortage, flooding, freezing, as well as organic matter physical and chemical protection (review in Davidson and Janssens 2006). Here, we test the hypothesis that the temperature sensitivity of decomposition of N-poor substrates, when mixed with N-rich litter, increases because the N shortage condition is relaxed. Organic matter decomposition in field conditions mainly depends on organic matter quality, water availability and temperature (Berg and McClaugherty 2008). In this study, to isolate the effects of temperature and litter quality, while minimizing the variability related to water availability, the decomposition experiment was carried out in microcosms under controlled conditions.

Based on the aforementioned considerations, four main hypotheses were tested: (1) in mixtures, N-rich substrates promote mass loss of N-poor materials; (2) a net N transfer occurs from N-rich to N-poor substrates; (3) temperature sensitivity of decomposition is enhanced when N-poor substrates are mixed with N-rich litter; and (4) C biochemical quality of N-poor substrates changes faster when mixed with N-rich litter.

Materials and methods

Plant material collection

Four organic substrate types were selected, showing very different values of C/N ratio, and percent N and lignin content, as follows (values are average \pm standard deviations): i) *Hedera helix* leaf litter, a fast decomposing evergreen vine (N content= 2.02 ± 0.12 %; C/N ratio= 21 ± 2.11 ; lignin content= 5.7 ± 0.76 %); ii) *Quercus ilex* leaf litter, a slow decomposing evergreen tree (N content= 1.24 ± 0.21 %; C/N ratio= 31 ± 3.43 ; lignin content= 18.3 ± 2.91 %) (Bonanomi et al. 2013); iii) woody sticks cut from *Q. ilex* branch (N content= 0.10 ± 0.02 %; C/N ratio= 440 ± 32.31 ; lignin content= 34.6 ± 4.54 %); and iv) cellulose strips (N content= 0.09 ± 0.01 %; C/N ratio= 466 ± 21.43 ; lignin content= 0.00 %). Leaf litter materials were sampled from a natural old-growth holm oak forest located on the

southern slope of Mt. Vesuvius (Portici 40° 48' 43" N – 14° 20' 49" E, Southern Italy). Freshly abscised leaves of each species were collected from plants randomly selected at the sampling site (n° plants >20), air dried for 20 days until reaching constant weight and stored afterwards at room temperature.

Decomposition experiment

Microcosms consisted of 300 mL polycarbonate Magenta vessels (Sigma-Aldrich, Co. LLC.) with polypropylene aerated lid. All leaf litter discs, woody sticks and cellulose strips were cut by scissors in order to obtain pieces of the correct mass (100 mg). Every batch was filled with 2 pieces of each material (i.e. experimental units). The organic substrates were introduced into microcosms and twice autoclaved. A microbial inoculum was prepared by mixing 90 g of water with 10 g of soil taken from the top 10 cm layer at the fields where litter was collected. The inoculum was sprayed inside the microcosms in order to improve the start up of the decomposition process. Ten types of experimental units were prepared, each consisting of 2 pieces of either the same organic substrate (4 types) or one of all the possible pairs of different substrates (6 combinations with a loading ratio of 50:50, i.e. a single piece for each materials). In most of previous decomposition experiments mass loss and nutrient dynamics were assessed in lumped mixed substrates, thus obscuring the response of different litter components. Here, instead, we kept separated the mixture components, making possible to assess potential species-specific effects between the different substrate types. The two pieces in each experimental unit were placed at distance of ~10 mm and were never in direct contact. Such spatial arrangement without direct contact between the two pieces, prevented the passive nutrient transfer between different organic substrates (Supplementary Fig. S1). Environmental conditions included not limiting water availability, obtained by watering the experimental units every three days with sterile distilled water until organic substrates were fully soaked, but avoiding connection by moisture between the two pieces in substrate pairs during the experiment, thus preventing nutrient leaching. However, other mechanisms of nutrient transfer between the two substrates in each experimental unit would still be possible, as for example a microbial colonization of the free space in between, creating a bridge between different litter types.

The full experimental design included 10 treatments incubated at two temperatures (12 and 24 °C) and replicated ten times for each of five dates of retrieval after incubation, for a total of 1,000 experimental units. Substrates were retrieved after 10, 30, 90, 180 and 360 days of decomposition. Collected substrates were oven-dried (60 °C until constant weight was reached) and weighted afterwards to the nearest 0.001 g.

Litter chemical analyses

Undecomposed materials were characterized for total C and N content by flash combustion of microsamples (5 mg of litter) in an Elemental Analyser NA 1,500 (Carlo Erba Strumentazione, Milan, Italy). Proximate cellulose and lignin content were quantified as acid hydrolysable fraction and acid unhydrolysable materials, respectively (Gessner 2005). C and N content of materials decomposing in the experimental units were also assessed, separately for each piece of organic substrate, after 90, 180 and 360 days of decomposition. Due to budget limitations, analyses were limited to C and N, omitting other relevant nutrients (e.g. P, K, Ca, etc.).

In order to assess changes of biochemical quality in the organic substrates, undecomposed materials, as well as materials decomposed for 360 days, were characterized by ^{13}C -CPMAS NMR (Kögel-Knabner 2002) obtained in solid state and under the same conditions, thus allowing a comparative analysis of the resulting spectra. The spectrometer used was a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe (for further details see Bonanomi et al. 2013). Spectral regions and corresponding C types have been identified following Bonanomi et al. (2011): 0–45 p.p.m. = alkyl C; 46–60 p.p.m. = methoxyl and N-alkyl C; 61–90 p.p.m. = O-alkyl C; 91–110 p.p.m. = di-O-alkyl C; 111–140 p.p.m. = H- and C- substituted aromatic C; 141–160 p.p.m. O-substituted aromatic C (phenolic and O-aryl C); 161–190 p.p.m. carboxyl C. The alkyl C/O-alkyl C ratio (0–45/61–110), considered a robust indicator of the degree of litter decomposition, was calculated following Almendros et al. (2000).

Data analysis

Generalized linear models (GLMs) were used to analyze the results of the decomposition experiments. For each type of organic substrate in the experimental units, main and second-order interactive effects of three

experimental factors were tested, considering percent mass remaining, N percent content, and C/N ratio as dependent variables. The type of paired substrate, temperature of incubation, and decomposition time were considered as treatment factors.

Nitrogen transfer was indirectly assessed by quantifying N content in each piece of organic substrate in all the different mixture combinations. In detail, during decomposition, an increase of total N content in a single organic piece, with a corresponding decrease in the piece to which it was paired, was used as evidence of N transfer between the two pieces.

The temperature sensitivity of organic substrates was estimated by calculating Q_{10} value (rate of change in the rate of mass loss given by a 10 °C change in temperature). Pair-wise differences at each level of treatment were statistically evaluated by post-hoc Duncan test. Significance was evaluated in all cases at $p < 0.05$.

Results

Litter mass loss and temperature sensitivity

In microcosms with monospecific substrates, decomposition was very rapid for *H. helix*, intermediate for *Q. ilex* and very slow for woody sticks and cellulose strips (Fig. 1). Mass loss significantly increased with temperature for all materials (Table 1). Mass loss significantly increased with temperature for all materials, with Q_{10} values (average \pm standard deviation) of 1.18 ± 0.12 , 1.35 ± 0.11 , 1.07 ± 0.17 , 1.10 ± 0.12 for *H. helix*, *Q. ilex*, woody sticks and cellulose strips, respectively.

Additive mass loss effects were found for both litters of *H. helix* and *Q. ilex*, in all mixtures at both incubation temperatures (Fig. 1). In other words, leaf discs decomposed at their own rates, independent of the other substrate in the mixtures (Table 1). Additive effects were also found for mass loss of paired cellulose strips and woody sticks (Fig. 1). In contrast, significantly accelerated decay rates (i.e. synergistic interaction) were observed for both cellulose strips and woody sticks when paired with *Q. ilex* and *H. helix* leaf litter (Fig. 1). Overall, no antagonistic interaction was recorded. The magnitude of synergistic interactions was outstanding. In particular, after 360 days of incubation at 24 °C cellulose strips lost 7.1 % of initial mass when unmixed and 6.2 % when paired with woody sticks, but 72 and 65 % when paired with *H. helix* and *Q. ilex*, respectively.

In the same conditions, woody sticks halved their mass when combined with *Q. ilex* and *H. helix* (mass loss equal to 45 and 50 %, respectively), while losing only 14 % when paired with cellulose strips, and 9 % when unmixed. Synergistic interactions occurred at both incubation temperatures but were enhanced at 24 °C. While pairing woody sticks with the two leaf litters did not produce significantly different mass loss dynamics, cellulose strips decomposed faster when combined with *H. helix* compared to *Q. ilex* (Fig. 1). Such different patterns were much more evident at 24 °C and over the first half of the decomposition period, and then converging towards similar values of mass remaining. In contrast, at lower temperature mass loss dynamics of cellulose strips paired with *H. helix* and *Q. ilex* progressively diverged throughout the observation period, producing significantly different values after 360 days (Fig. 1). Organic substrates showed different temperature sensitivity. Q_{10} values of *H. helix* and *Q. ilex* leaves were not significantly affected by the paired organic materials (Table 2). In contrast, woody sticks and cellulose strips showed a significantly higher temperature sensitivity when paired with plant leaf litter, compared to other mixtures (Table 2).

N dynamics and transfer between organic substrates

In monospecific microcosms N concentration significantly increased (Fig. 2) and C/N ratio decreased (Fig. 3) during the 360 days of decomposition for all organic substrates.

Concerning N content dynamics in the tested materials, *H. helix* leaf discs showed additive responses in all mixtures at both incubation temperatures (Fig. 2, Supplementary Table S1). In other words, for *H. helix* increases of tissue N concentration were independent of the other substrate in the microcosm. A similar pattern of additive interactions was found in the case of *Q. ilex* leaf discs, but with the important difference of a faster N content increase when paired with *H. helix* litter, compared to the other materials (Fig. 2, Supplementary Table S1). Cellulose strips showed additive interaction when paired with woody sticks, but remarkable synergistic interactions when combined with both leaf litter types (Fig. 2, Supplementary Table S1). The increase of N concentration in cellulose and wood when combined with N rich litters was evident by the rapid decrease of the C/N ratio during the whole experiment. In the case of cellulose paired with leaf discs, after 360 days of

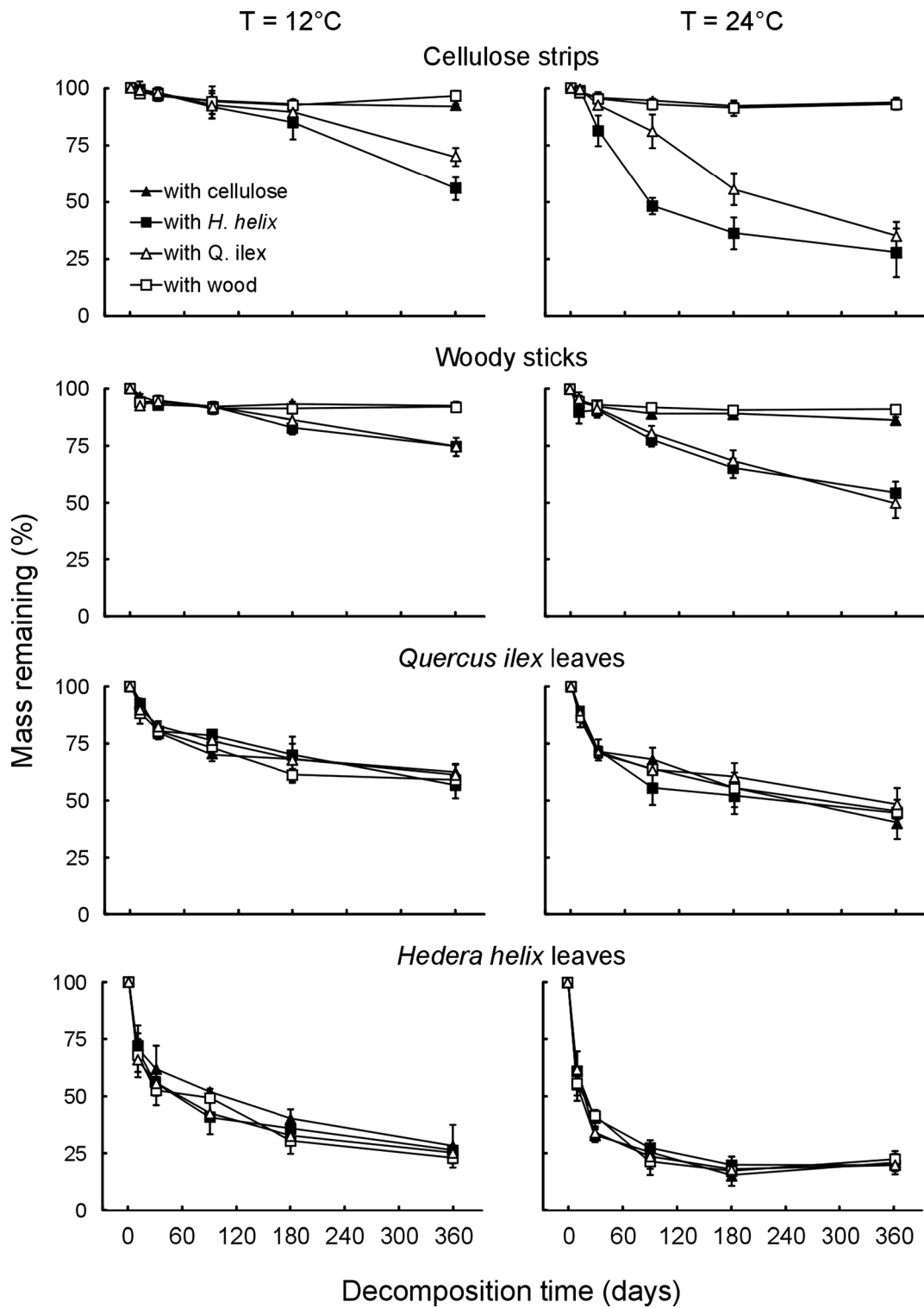


Fig. 1 Decomposition dynamics in experimental microcosms. Variations of mass remaining in cellulose, wood, and leaf litters of *Quercus ilex* and *Hedera helix* decomposing for 360 days at two

temperatures, paired with four different organic substrates. Data refer to mean \pm standard deviation of 10 replicates for each material

Table 1 Generalized Linear Models (GLMs) for mass remaining, N content and C/N ratio. GLMs for four types of organic substrate (cellulose strips, woody sticks, leaf litters of *Quercus ilex* and *Hedera helix*) decomposing in presence of different additional

substrates. Data refer to sum of squares (SS), degrees of freedom (df) and F statistics for main and second order interactive effects of type of additional substrate (S), decomposition time (D), and incubation temperature (T) on the tested dependent variables

		Mass remaining (%)				Nitrogen content (%)				C/N ratio			
		SS	Df	F	p	SS	df	F	p	SS	df	F	p
Cellulose	Type (S)	1114309	3	63.62	<0.001	2.316	3	16.94	<0.001	286350	3	82.17	<0.001
	Time (D)	1225148	4	52.46	<0.001	1.521	2	16.76	<0.001	145676	2	62.71	<0.001
	Temperature (T)	411899	1	70.55	<0.001	0.353	1	7.79	0.009	1465	1	1.26	0.270
	S × D	920572	12	13.14	<0.001	1.049	6	3.85	0.006	10266	6	1.47	0.220
	S × T	380876	3	21.75	<0.001	0.618	3	4.54	0.009	44370	3	12.73	<0.001
	D × T	172403	4	7.38	<0.001	0.114	2	1.25	0.299	3550	2	1.53	0.233
Wood	Type (S)	310565	3	66.28	<0.001	0.790	3	50.72	<0.001	347027	3	118.31	<0.001
	Time (D)	478262	4	76.55	<0.001	0.524	2	50.49	<0.001	262854	2	134.42	<0.001
	Temperature (T)	123232	1	78.90	<0.001	0.001	1	0.06	0.811	8566	1	8.76	0.006
	S × D	298729	12	15.94	<0.001	0.298	6	9.57	<0.001	27602	6	4.71	0.002
	S × T	68407	3	14.60	<0.001	0.061	3	3.92	0.018	17952	3	6.12	0.002
	D × T	65173	4	10.43	<0.001	0.021	2	2.05	0.147	3667	2	1.87	0.171
Q. ilex	Type (S)	12237	3	1.91	0.134	0.578	3	12.71	<0.001	54.79	3	4.30	0.012
	Time (D)	1931537	4	225.70	<0.001	1.660	2	54.79	<0.001	194.86	2	22.96	<0.001
	Temperature (T)	290329	1	135.70	<0.001	1.593	1	105.19	<0.001	89.44	1	21.08	<0.001
	S × D	15175	12	0.59	0.844	0.413	6	4.54	0.002	20.45	6	0.80	0.575
	S × T	9381	3	1.46	0.230	0.680	3	14.96	<0.001	95.83	3	7.53	<0.001
	D × T	49585	4	5.79	<0.001	0.012	2	0.41	0.666	47.19	2	5.56	0.009
H. helix	Type (S)	13152	3	1.43	0.239	0.989	3	1.27	0.295	22.442	3	3.42	0.024
	Time (D)	2733392	4	223.07	<0.001	3.302	2	6.36	0.003	82.585	2	18.85	<0.001
	Temperature (T)	674925	1	220.32	<0.001	5.073	1	19.54	<0.001	55.795	1	25.48	<0.001
	S × D	31388	12	0.85	0.596	0.340	6	0.22	0.969	4.749	6	0.36	0.899
	S × T	29012	3	3.16	0.028	1.133	3	1.45	0.238	7.651	3	1.16	0.332
	D × T	121036	4	9.88	<0.001	3.433	2	6.61	0.003	24.397	2	5.57	0.006

decomposition the C/N ratio decreased to values below or just above 100, while being more than double when

the strips were unmixed, or paired with woody sticks (Fig. 3, Supplementary Table S2). A similar pattern was

Table 2 Temperature sensitivity estimated by Q_{10} index (values are average ± standard deviations) calculated for different organic substrate (cellulose strips, woody sticks, leaf litters of *Quercus ilex* and *Hedera helix*) decomposed in presence of different paired

organic materials across the whole decomposing period of 360 days. Different letter indicate statistically significant differences within each organic matter type (ANOVA Duncan test; $p < 0.05$)

Substrate type	Paired organic substrate			
	Cellulose	Wood	Quercus ilex	Hedera helix
Cellulose	1.10±0.12 a	1.22±0.14 a	2.56±0.15 b	3.84±0.21 c
Wood	1.39±0.13 b	1.07±0.12 a	1.81±0.11 c	1.72±0.13 c
Quercus ilex	1.29±0.18 a	1.31±0.17 a	1.35±0.18 a	1.47±0.22 a
Hedera helix	1.36±0.21 a	1.19±0.18 a	1.22±0.15 a	1.18±0.17 a

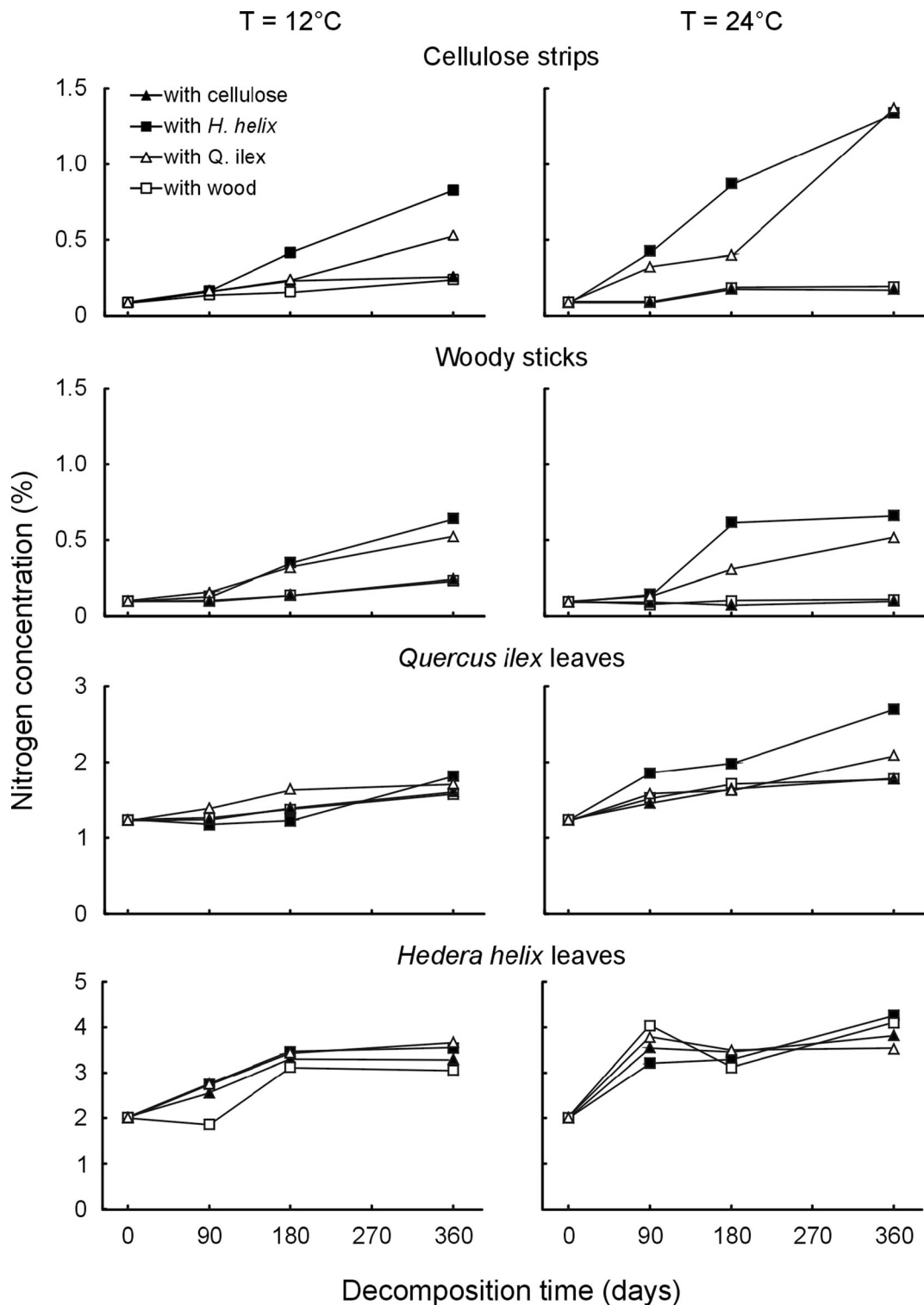


Fig. 2 Nitrogen content variation in litter mixtures. Dynamics of N concentration in cellulose, wood, *Quercus ilex* and *Hedera helix* litter decomposing for 360 days at two temperatures, paired with four different organic substrates. Data refer to mean of 10

replicates for each material. Note different scales on Y axis. Standard deviation bars are not reported to improve readability. Descriptive statistics and testing for significant differences are in Supplementary Table S1

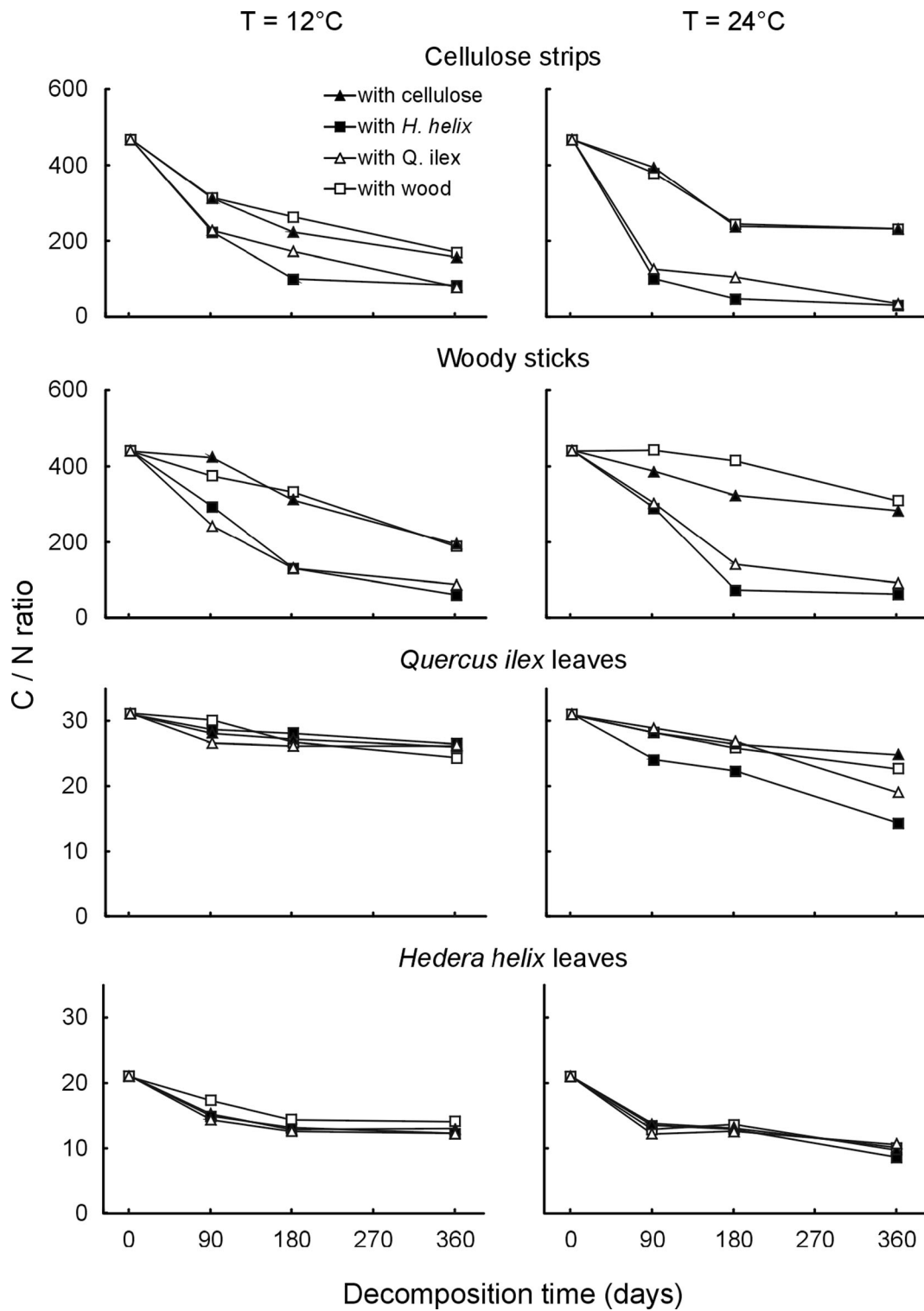


Fig. 3 C/N ratio dynamics in litter mixtures. Changes of C/N ratio in cellulose, wood, *Quercus ilex* and *Hedera helix* leaf litter decomposing for 360 days at two temperatures, paired with four different organic substrates. Data refer to mean of 10 replicates for

each material. Note different scales on Y axis. Standard deviation bars are not reported to improve readability. Descriptive statistics and testing for significant differences are in Supplementary Table S2

observed in the case of woody sticks, showing a C/N ratio as much as three times lower when paired with leaf discs, compared to sticks unmixed or paired with cellulose strips (Fig. 3, Supplementary Table S1).

Total N content after 360 days of decomposition was lower than the initial value for the two plant litter types, independent of the additional substrate in the mixture, indicating a net N loss (Fig. 4). N release, with values around 50 % of the initial N content, was more prominent for *H. helix* compared to *Q. ilex* (Fig. 4). Total N content of cellulose and wood was remarkably different in relation to the additional substrate in the mixture (Fig. 4). In fact, at both incubation temperatures, initial N amount remained almost unchanged when the cellulose and wood materials were unmixed or paired together. In contrast, the final N amount was much higher when such materials were paired with the two leaf litters, indicating a net N immobilization (Fig. 4). Our experiment was specifically planned to control two possible mechanisms of N transfer between different litter types, such as passive diffusion and leaching, but not to precisely assess other possible concurrent mechanisms. In this regard, it is noteworthy that bridges of fungal hyphae rapidly appeared (within 7 days) in the space in

between two pieces of material with very different N content, (G.B., personal observation; Supplementary Fig. S2) but not in the pairs of substrates of the same type.

Changes of biochemical C quality in organic substrates

The four undecomposed substrates showed remarkably different ^{13}C NMR signals. In detail, cellulose and wood spectra, compared to the two leaf litter types (Bonanomi et al. 2013) showed higher peaks at 61–90 p.p.m., and lower at 0–45 p.p.m., corresponding to O-alkyl C and alkyl C regions, respectively.

After 360 days of decomposition, all the tested materials showed a general trend of decreasing O-alkyl C, mainly associated to sugars and polysaccharides, and increasing alkyl C, related to lipid waxes, cutins, and microbial products as methoxyl and N-alkyl C related to lignin (46–60 p.p.m.). Signals in the, in the di-O-alkyl C (91–110 p.p.m.) and in the carboxyl C (161–190 p.p.m.) regions, as well as those in the two aromatic C regions (111–140 p.p.m. and 141–160 p.p.m.) did not change significantly. However, in the cases of *H. helix* and *Q. ilex* litters, such biochemical changes were

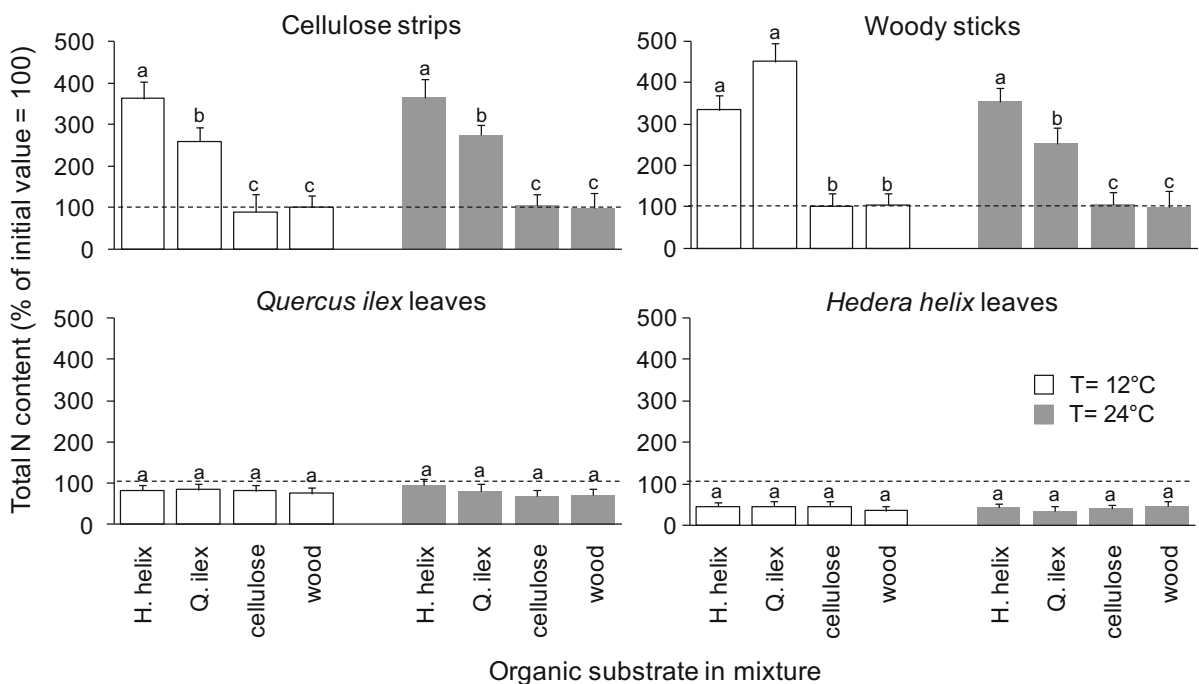


Fig. 4 Nitrogen transfer in litter mixtures. Nitrogen content after 360 days of decomposition at 12° and 24 °C, expressed as percentage of initial value (=100 %, indicated by dashed lines) in wood, cellulose, and leaf litters of *Hedera helix* and *Quercus ilex*,

paired with four organic substrates. Data refer to mean \pm standard deviation, different letters indicate statistically significant differences within each organic matter type and incubation temperature (Duncan test; $p < 0.05$)

unaffected by the type of additional substrate present in the mixture (data not shown), whereas for cellulose and wood the observed variations were highly mixture-dependent. In particular, the decrease of *O*-alkyl-C and the increase of alkyl-C peaks were limited when such materials were paired with N poor substrates, and more rapid in mixtures with N rich leaf litters (Fig. 5).

Accordingly, the indicator of stability calculated as the ratio alkyl-C/*O*-alkyl-C increased for all materials after 360 days of decomposition. Again, in the cases of

cellulose and wood, but not for *H. helix* and *Q. ilex*, the rate of increase of the indicator was highly affected by the pair composition in the experimental units. In detail, cellulose and wood showed very low initial values of the indicator (0.013 and 0.021, respectively), and a relatively slow increase when the materials were either unmixed (final values of the indicator equal to 0.024 and 0.104) or paired together (0.042 and 0.043). Conversely, the alkyl C/*O*-alkyl C ratio increased more rapidly in the combinations cellulose-*Q. ilex* litter (0.057) and

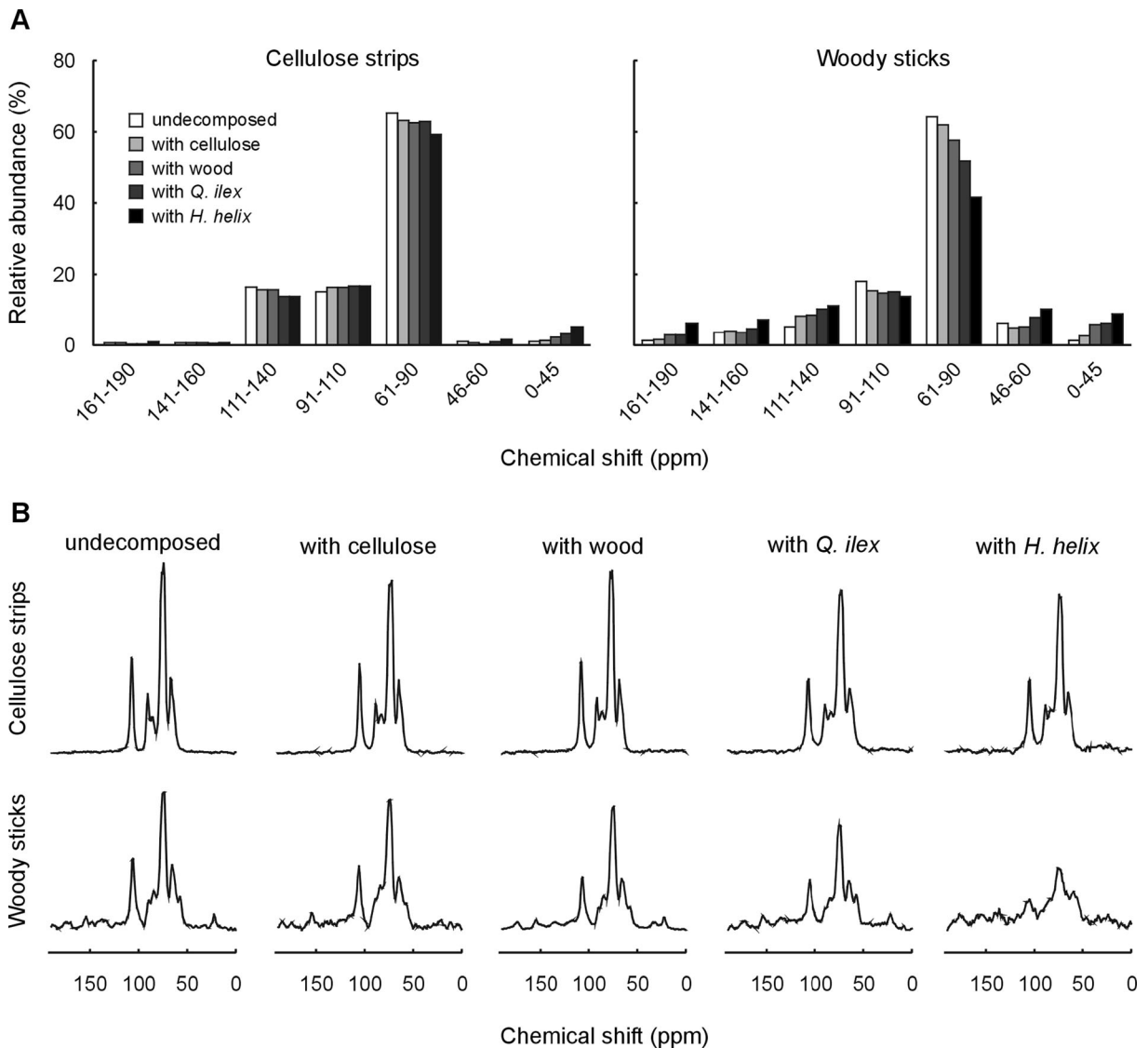


Fig. 5 Variation of C biochemical quality during decomposition. **a** Relative abundance of seven main classes of organic C assessed by ^{13}C -CPMAS NMR spectroscopy in cellulose strips and woody sticks either undecomposed or after 360 days of decomposition at

24 °C, paired with four different organic substrates. **b** Spectra of cellulose strips and woody sticks undecomposed or after 360 days of decomposition paired with four different organic substrates

cellulose-*H. helix* litter (0.093), being even faster for wood paired with *Q. ilex* (0.123) and *H. helix* (0.216) litters.

Discussion

Our experiment, based on four organic substrates representing a broad range of biochemical quality, demonstrated that the decomposition of mixed residues is affected by additive as well as synergistic, non-additive responses. We showed that mass loss, N dynamics, sensitivity to temperature and changes in C biochemical quality are regulated by the N content of the mixture components. Specifically, additive effects on mass loss and N dynamics were observed when substrates with similarly high (*H. helix* and *Q. ilex*) or low (cellulose and wood) N content were paired. In contrast, synergistic interactions were found when substrates with contrasting N content were associated (either of the two leaf litters with either cellulose or wood). Moreover, decomposition of N poor substrates (cellulose and wood) paired with N rich materials showed a higher temperature sensitivity and faster changes of C quality, compared to unpaired substrates, likely as a result of N shortage relaxation. On the other hand, temperature sensitivity and C quality changes of N rich litter (*H. helix* and *Q. ilex*) were unaffected by mixing. Overall, no antagonist effects were observed in this experiment. Our findings support the hypothesis that the occurrence in mixtures of high quality litters, with low C/N ratio and high N content, promote mass loss and organic C changes of other, poor quality materials, through N transfer.

Three literature reviews focused on this issue, reporting that litter mixing had mostly non-additive effects on mass loss, with a prevalence of synergistic over antagonistic responses (Gartner and Cardon 2004; Hättenschwiler et al. 2005; Lecerf et al. 2011). In our experiment, non-additive synergistic effects were the rule when mixtures were composed of substrates with contrasting N content. Synergistic interactions among decomposing materials have been often attributed to a net transfer of nutrients from a source litter to a target, usually nutrient-poor substrate (Chapman et al. 1988). Our study, indeed, provides a direct support to such model. In fact, we found an increase in N content in all cases of N-poor materials whenever they were paired with N-rich litters, coupled with a decrease of N content

in the latter. This is consistent with a net transfer of N from plant litter to wood and cellulose, which explains the consequent increase of the mass loss rate of the target materials.

Accordingly, the observed pattern of decreasing C/N ratio for all tested materials fits well with the N transfer model. In fact, decaying organic matter releases carbon at a faster rate than N, so that C/N ratio is expected to progressively decrease until a threshold value is attained. Here, C/N ratio of cellulose and wood decreased faster in presence of N-rich litters, likely as a result of N transfer from such sources. N transfer among decomposing litters in mixture has been previously reported (e.g. Schwendener et al. 2005; Schimel and Hättenschwiler 2007). However, the resulting mass loss of mixed litters showed different responses. For instance, Lummer et al. (2012) reported a remarkable transfer of N between litters of the nutrient poor beech (*Fagus sylvatica*) and the nutrient rich ash (*Fraxinus excelsior*), but the resulting mass loss was additive. In other cases, mixing N poor with N rich litters resulted in non-additive antagonistic interactions (e.g. Bonanomi et al. 2010; Tan et al. 2013). Nevertheless, it is important to keep in mind that our experimental setup was different from the previous studies, which were carried out with litter-bags placed in direct physical contact with the soil. In this way, alternative sources of N might have been provided, such as N release by soil microbes. Previous results contrast with the outstanding non-additive, synergistic interactions observed in this study at both incubation temperatures. However, such contrasting results are not unexpected, and could be partially attributed to the broader range of N content in the materials used in this experiment, varying from 2.02 % of *H. helix* to <0.1 % of cellulose and wood, compared to previous studies. Interestingly, we found that the mass loss of the putative N source litters (both *H. helix* and *Q. ilex*) remained unaffected by the mixture composition. Such evidence does not support the hypothesis by Berglund and Ågren (2012), suggesting that in two-litter mixtures one litter can decompose faster while the other component can show a slower decomposition compared to unmixed samples. On the other hand, as predicted by the Berglund and Ågren (2012) model, the observed positive effect of N sources was ranked according to their N mineralization rates, being highest for *H. helix* litter, intermediate for *Q. ilex*, and null for cellulose and wood that only immobilized N. However, evidence available from the literature is not conclusive.

Recently, comparing different pathways of N transfer between two litter species of contrasting N status, Lummer et al. (2012) reported that the transfer was larger for the N poor beech than for the N rich ash, and interpreted such surprising result with the selective development of fungal (more able to transfer N) and bacterial (less able to transfer N) dominated microbial communities over decomposing litter of beech and ash, respectively.

The different responses of litter mass loss recorded in this study can be related also to the C quality of the substrates and, with more detail, to their initial lignin content. External supply of inorganic N, as well as high litter N content, can inhibit mass loss as a result of reduced lignin degradation (Berg and McLaugherty 2008). In fact, it is known that N has a dual, opposite effects on litter decomposition, promoting litter mass loss during the early stages (up to 30–40 % of mass loss) and limiting it thereafter (Berg and Matzner 1997; Bonanomi et al. 2013). In the early decay phases, high N availability may sustain microbial activity to rapidly consume labile C compounds, resulting in a rapid mass loss rate. In contrast, high N concentration in the later stages inhibits mass loss by favoring the formation of recalcitrant chemical complexes with lignin (Hatakka 2001), by inhibiting oxidative enzymes involved in lignin degradation, or reducing the multiple functionality of litter decomposer microbial communities (Hobbie et al. 2012). In our study, N transfer from high quality litter stimulated mass loss of both lignin devoid (cellulose) and lignin rich (wood) substrates throughout the decomposition period (360 days). Such evidence apparently contradicts the previous studies, as well as the findings of a meta-analysis by Knorr et al. (2005) in which, on average, external addition of inorganic N forms reduced the decay rate of low quality, lignin rich substrates. In previous studies it has been showed that the inhibitory effect of N is most prominent at the latest stages of decomposition, usually when it lasts for more than 24 months (Knorr et al. 2005). Here, instead, the very low initial N content of cellulose strips and woody sticks, coupled with the absence of other N sources (i.e. no soil was included in microcosms) likely produced an extreme microbial N starvation, that eventually might have been relaxed once external N was provided. The transfer of N from leaf litter, especially of *H. helix*, to woody sticks, and the consequent enhancing effect on mass loss rate, bear importance for the decomposition of woody debris in natural woodlands, with potential

consequences at ecosystem level. The occurrence of N rich litter (e.g. the vine *H. helix* which is widespread in many woodland systems) coupled with their rapid N mineralization rate can provide a consistent nutrient flux that may sustain wood decomposition. Previous studies on decomposition of wood debris focused on the role of climatic conditions and substrate quality in terms of wood density, nutrient content and lignin quality (for a review see Weedon et al. 2008). Our results suggest that the variability of N availability at local scale, which is largely related to soil quality and litterfall patterns, might greatly affect wood debris decomposition. Further studies are needed to address the consistency and relevance of N transfer from different litter types to woody debris under field conditions. Our observations of a fungal bridge, connecting spatially separated litter pieces with very different N content, but not in other types of mixtures, suggests that N transfer may be mediated by fungal activity, at least in the tested experimental conditions. However, the occurrence of other mechanisms underlying N transfer, and whether they are active in nature, should be further investigated.

In this study, we observed the strongest synergistic interactions when mixed N-and N-poor substrates were decomposing under warmer conditions. This finding may be related to the fact that microbial, as well as enzymatic activities, are generally faster at higher temperatures, especially under no other ecological limitations. Temperature sensitivity of decomposing litter is a debated issue, with theoretical and empirical evidence suggesting an increasing sensitivity with substrate recalcitrance (Davidson and Janssens 2006; Hartley and Ineson 2008), although opposing evidence are available (e.g. Giardina and Ryan 2000). In our experiment, when decomposed alone, cellulose strips and woody stick showed a limited temperature sensitivity compared with *H. helix* and *Q. ilex* leaf litter. This result can likely be explained by the very limited mass loss achieved by cellulose and wood when decomposed alone as a result of the drastic N limitation. In contrast, decomposition rates of both woody sticks and cellulose strips, when mixed with N rich substrates, showed a sharp increase in temperature sensitivity. These results indicate that temperature sensitivity of N poor and/or recalcitrant substrates increases as the environmental conditions affecting litter decomposition (e.g. N availability) improve. Finally, temperature sensitivity of the two leaf litters were unaffected by the type of paired organic materials. In our knowledge, this is the first study that report a

significant effect of litter mixture on decomposition temperature sensitivity. This result is consistent with the hypothesis that temperature sensitivity of organic substrate can be reduced in presence of environmental constraints such as water and nutrient shortage, flooding, freezing, as well as organic matter physical and chemical protection (Davidson and Janssens 2006).

Concerning C quality, here, for the first time, we described biochemical changes of C types in different litter mixtures using ^{13}C NMR spectroscopy. The most interesting results are the very small spectral changes showed by both cellulose and wood when decomposed either unmixed or in mixture with other N poor materials, and the increase of such spectral variations when occurring in mixtures with *H. helix* and *Q. ilex*. The observed reduction of carbohydrates (O-alkyl C), the increase of alkyl C and the consequent small, but consistent increase of the alkyl C/O-alkyl C ratio indicate a progressive increase of organic matter stability (Almendros et al. 2000). Such rapid changes of C quality of cellulose and wood only in presence of N rich litter could be due to a more active microbial community, able to maximize the consumption of the carbohydrates fraction and allowing, even as a result of microbial by products and spoilage build up, a progressive accumulation of aliphatic compounds (Kögel-Knabner, 2002). Future studies in this direction can unravel if litter mixing has relevant implications for changes of litter C quality and, then, for ecosystem C storage.

Conclusions

Our results provided evidence that litter quality, in particular N content, is the most important factor affecting mass loss and nutrient dynamics in mixture, consistently with previous studies (Wardle et al. 2006; Lecerf et al. 2007). In the context of the debated effects of N on mixed litter decomposition (Hobbie et al. 2012) the finding of synergistic interactions between N poor and N rich substrates, as a result of N transfer and irrespective of the substrate lignin content, provides a new contribution. Future studies can explore the implications of our finding in the context of ecosystem functioning related to plant invasion, since it is well recognized that invasion often increases N pools and accelerates ecosystem N fluxes (Castro-Díez et al. 2014). Previous evidence showed that litter mixing effects are apparently idiosyncratic, both in terms of litter decay rate and

nutrient release. The present study indicates that, at least for materials with contrasting N content, additive and non-additive interactions are predictable based on initial N content and C/N ratio. Our study did not identify the mechanisms underlying N transfer, although the formation of fungal bridge connecting N rich to N poor substrates suggests that fungi may play an important role in this process. The variability of N effects on litter mass loss as well as the different temperature sensitivity of litter in mixtures indicate the utility of further studies on this issue, particularly when based on mixture component assessment.

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