Leaf Nutrients and Macroinvertebrates Control Litter Mixing Effects on Decomposition in Temperate Streams

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ABSTRACT

Plant litter decomposition is an essential ecosystem function in temperate streams. Both riparian vegetation and decomposer communities are major determinants of the decomposition efficiency and the interactions occurring within litter mixtures. However, the extent to which such litter mixture interactions are affected by combined shifts in litter traits and decomposer community is not well understood. We used leaf litter from 10 European tree species in order to study litter decomposition and litter mixture effects occurring in two-species litter mixtures in a temperate forested stream of northwestern France. The study distinguished between (i) decomposition involving microorganisms alone or together with invertebrates, and (ii) decomposition involving litter mixtures of similar or dissimilar nutrient content. Increasing mean litter nutrient concentration favored both microbial activity and litter decomposition rate. Surprisingly, the highest litter mixture effects occurred in mixtures containing two nutrient-rich litters and occurred mainly in macroinvertebrate presence. Both the "mass-ratio hypothesis", expressed as the community weighted mean traits ($Trait_{CWM}$), and the "niche complementarity hypothesis", expressed as the functional dissimilarity of litter traits ($Trait_{FD}$), contributed to explain litter mixture effects. However, $Trait_{CWM}$ was found to be a better predictor than $Trait_{FD}$. Finally, when evaluating the individual contributions of litter nutrients, calcium and magnesium appeared as important drivers of litter mixture effects. Our findings suggest that the mass-ratio hypothesis overrules the niche complementarity hypothesis as a driver of litter diversity effects. Our study highlights the key importance of macroinvertebrates and of leaf nutrients, such as Ca and Mg, which are often neglected in decomposition studies in streams.

KEYWORDS: Biodiversity-ecosystem functioning; community weighted means; functional trait dissimilarity; litter traits; litter decomposition; litter nutrients; temperate stream

HIGHLIGHTS

- Macroinvertebrates and leaf nutrients drive microbial activity and litter decomposition rate
- Macroinvertebrate presence and nutrient concentration control litter mixing effect intensity
- Mean nutrient concentration is more important than concentration dissimilarity within litter mixture

INTRODUCTION

Litter decomposition is an essential ecosystem function controlling the carbon (C) and nutrient cycles in both terrestrial (Swift and others 1979; Cadish and Giller 1997) and aquatic (Cummins 1974; Wallace and others 1997) ecosystems. Litter decomposition rate is jointly affected by the litter traits (e.g. Cornwell and others 2008; Garcia-Palacios and others 2016), the environmental conditions, such as the temperature (e.g. Fierer and others 2005; Follstad Shah and others 2017), and the decomposer communities (e.g. Hättenschwiler and others 2015; Gessner and others 2010; Jabiol and others 2013; Santonja and others 2018a). Rates of litter decomposition are essentially controlled by litter chemistry (Cornwell and others 2008; Garcia-Palacios and others 2016). In addition to secondary compounds (e.g. tannins) and fiber components (e.g. lignin), concentrations of nutrients such as nitrogen (N) and phosphorus (P) are useful litter traits for the prediction of the decomposition rates of single-species litter (Gessner and Chauvet 1994; Cornwell and others 2008; Schindler and Gessner 2009). Nevertheless, as mainly reported in terrestrial ecosystems, other nutrients such as calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), and sulfur (S), can also affect the decomposition efficiency (Kaspari and others 2008; Makkonen and others 2012; Garcia-Palacios and others 2016). However, the potential importance of these nutrients is often neglected in decomposition studies in streams compared to N and P.

In the majority of natural ecosystems, litter material from different plant species decomposes together. Given that forested stream food webs are dependent upon allochthonous leaf litter (Vannote and others 1980; Wallace and others 1997; Gessner and others 1999), understanding the relationship between riparian tree species composition (and its associated litter traits) and the decomposition rates in streams draining forested watersheds is of considerable ecological importance (Swan and Palmer 2004; Kominoski and others 2007). Changing plant species composition can modify litter trait control over decomposition in two

ways. First, according to the "mass-ratio hypothesis" (Grime 1998; Garnier and others 2004), litter trait control over decomposition changes along community-weighted mean (CWM) trait values (Fig. 1a, Quested and others 2007; Mokany and others 2008; Laughlin 2011). Second, according to the "niche complementarity hypothesis" (Petchey and Gaston 2006; Diaz and others 2007), a change in the functional dissimilarity (FD) of litter trait-values affects the degree to which resource-use complementary occurs within the decomposer community, and its capacity to induce non-additive litter mixing effects on decomposition (i.e. litter mixtures that decompose at different rates than predicted by the mass-ratio hypothesis) (Fig. 1b, Wardle and others 1997; Vos and others 2013; Handa and others 2014). These two mechanisms can operate simultaneously by affecting both decomposer community and decomposition efficiency as a result of plant (litter) composition change (e.g. Garcia-Palacios and others 2017; Santonja and others 2018b). However, our knowledge about the relative importance of these two mechanisms in controlling the decomposer communities and the efficiency of the decomposition process in streams is very limited.

Mechanisms involved in positive litter mixing effects likely include nutrient transfer, such as N or P, from nutrient-rich to nutrient-poor litter species. For example, nutrients may be transported through fungal hyphae connecting two different leaf litter species (Schimel and Hättenschwiler 2007; Gessner and others 2010; Lummer and others 2012), in which case an acceleration of decomposition in species mixtures is expected since the nutrients are used more efficiently overall. According to the literature (Wardle and others 1997; Sanpera-Calbet et al. and others 2009; Santonja and others 2015a, 2015b; Santschi and others 2018), positive mixing effects are most likely expected in the mixtures including one poor-quality litter species and a high-quality one (Fig 1b; but see Frainer and others (2015) for contrasting results). However, whether, and to what extent, mixing litter affects decomposition rates in streams remains much debated, since litter mixture effects appear to be idiosyncratic (Schindler and Gessner 2009;

99 Gessner and others 2010; Cardinale and others 2011; Lecerf and others 2011). Previous studies
100 highlighting contrasting results have focused on the effects of N and P in explaining such litter
101 mixture effects (e.g. Frainer and others 2015; Santschi and others 2018). However, other key
102 nutrients, such as Mg or Ca, could potentially affect decomposition rates in litter mixtures.
103 Additionally, it is also not clear to what extent macroinvertebrates contribute to
104 decomposition in litter mixtures (Hättenschwiler and Gasser 2005; Swan and Palmer 2006b;
105 Swan 2011). Shredder macroinvertebrates can play a prominent role in the decomposition
106 process in stream ecosystems (Anderson and Sedell 1979; Cummins and Klug 1979; Handa and
107 others 2014; Garcia-Palacios and others 2016), by consuming and fragmenting the litter
108 material (Allan 1996; Graça 2001), by stimulating microbial decomposition (Wetzel 1995;
109 Villanueva and others 2012), and by mediating plant diversity effects on litter decomposition
110 (Lecerf and others 2005; Kominoski and others 2007; Sanpera-Calbet and others 2009).
111 According to Cummins and others (1989), such a role played by macroinvertebrates would be
112 higher in litter mixtures of distinct degradability than in more homogeneous litter mixtures.
113 Indeed, this type of litter mixture could simultaneously create a suitable microhabitat (more
114 refractory litter) and a food resource (more labile litter). Additionally, litter mixtures could
115 promote an increase in both growth rate (Swan and Palmer 2006a) and macroinvertebrate
116 population due to their aggregative behavior (Presa-Abós and others 2006), enhancing therefore
117 the decomposition of litter mixtures (Sanpera-Calbet and others 2009).
118 In order to address these gaps, we used leaf litter from 10 common European tree species
119 to evaluate how litter diversity effects are affected by litter nutrients (Ca, K, Mg, N, Na, P, and
120 S) in a temperate stream. Moreover, in order to elucidate which organisms may be responsible
121 for mediating such litter diversity effects, we distinguished between decomposition involving
122 microorganisms alone (with decomposition in fine-mesh litterbags) and both microorganisms
123 and macroinvertebrates (with decomposition in coarse-mesh litterbags) (Boulton and Boon

1991). Five of the plant species showed nutrient-rich litter, while the other five showed nutrient-poor litter (Fig. 2). Mixtures of two litter species were created in all possible pairwise combinations, in order to distinguish between litter mixtures of similar nutrient content (i.e. in mixing two nutrient-poor or two nutrient-rich litters) and litter mixtures of dissimilar nutrient content (i.e. in mixing one nutrient-poor litter and one nutrient-rich litter). Despite the fact that both litter traits, litter mass loss and microbial activity are continuous variables, such artificial distinction into two discrete nutrient categories was necessary to better understand where and why litter diversity effects occur. We evaluated (i) the litter decomposition rate and the microbial activity (i.e. CO_2 production) in the two-species litter mixtures, and (ii) the litter diversity effects occurring on the decomposition rate and on the microbial activity in these litter mixtures after 30 days of immersion in a temperate forested stream in northwestern France (Piscart and others 2009).

First, according to the "mass-ratio hypothesis" (Grime 1998; Garnier and others 2004), the increase in both litter decomposition rate and microbial activity would be positively correlated to the increase in CWM scores in nutrient concentration within the litter mixture (Cornwell and others 2008; Foucreau and others 2013). Therefore, in our first hypothesis, we predicted higher litter decomposition rate and microbial activity in the mixture of two nutrient-rich litters (Fig. 1a). Second, according to the "niche complementarity hypothesis" (Petchey and Gaston 2006; Diaz and others 2007), mixtures of very dissimilar litter species yield the highest FD scores and are related to higher litter diversity effects (Wardle and others 1997; Lecerf and others 2011; Santschi and others 2018). Thus, in our second hypothesis, we predicted the highest litter diversity effects in the mixtures of nutrient-poor and nutrient-rich litters (Fig. 1b), which would be shown by an increase in litter decomposition rate and microbial activity due to synergistic effects between the two litters in mixtures in the present study. Finally, macroinvertebrates may contribute up to 6 times more influence than microorganisms

149 on litter decomposition in temperate unaltered forested streams (Piscart and other 2009), favor
150 microbial community development (Wetzel 1995; Villanueva and others 2012), and mediate
151 litter diversity effects (Lecerf and others 2005), we hypothesized that litter decomposition rate,
152 microbial activity, and litter diversity effects would be enhanced by macroinvertebrate presence
153 (Figs. 1a and 1b).
154
155 **MATERIALS AND METHODS**
156
157 ***Study site and material collection***
158 The experiment was conducted in the Hermitage stream, located in the Villecartier
159 Forest in northwestern France (48°28' N, 1°33' W). The stream bed was dominated by sand
160 and leaf litter (site H1 in Piscart and others 2009). The stream water was circumneutral, well-
161 oxygenated, and possessed moderate nutrient concentrations (Piscart and others 2009): 11 ± 1
162 mg l^{-1} dissolved oxygen, 49 ± 18 µg N l^{-1} ammonium, 510 ± 19 µg N l^{-1} nitrate, 10 ± 8 µg N l^{-1}
163 nitrite, 19 ± 8 µg P l^{-1} phosphate. The macroinvertebrate community of the Hermitage stream
164 was composed of species belonging to the Amphipoda, Isopoda and Trichoptera orders, with
165 crustaceans representing 74% of this macroinvertebrate community (Supplementary Table S1).
166 *Fagus sylvatica* was the dominant tree species in the forested watershed, but the riparian
167 vegetation was composed of diverse deciduous tree species, including those employed in the
168 experiment.
169 The leaf litter of 10 common European tree species was collected: *Acer platanoides*,
170 *Alnus glutinosa*, *Betula pendula*, *Castanea sativa*, *Corylus avellana*, *Carpinus betulus*, *Fagus*
171 *sylvatica*, *Quercus robur*, *Salix atrocinerea* and *Tilia cordata*, hereafter referred to by their
172 genus name. These 10 species were selected using the existing literature (e.g. Lecerf and others
173 2007; Schindler and Gessner 2009; Santonja and others 2018a), based on the nutrient

concentrations of their leaves, to represent five species with nutrient-rich litter (NRL), and five with nutrient-poor litter (NPL) (Fig. 2; a cluster analysis well discriminates these two groups). Freshly abscised leaves were collected over the entire period of maximum litter fall, from October to November 2015. They were dried at room temperature, and stored until the beginning of the experiment.

Litter decomposition experiment

Leaf litter decomposition was studied for a period of 30 days, using the litterbag method (Boulton and Boon 1991). Coarse- and fine-mesh litterbags (5 mm and 0.5 mm mesh size, respectively) were used and filled with 2 g of dry leaves. The leaf litter enclosed in fine-mesh litterbags was only accessible to microorganisms, whereas the coarse-mesh bags also allowed access to macroinvertebrates. The litterbags contained either a single species (10 treatments) or a mixture of two species in all possible pairwise combinations (45 treatments). The mixed-species litterbags received equal amounts of both species (i.e. 1 g). A total of 440 litterbags ([10 single-species + 45 two-species mixtures] × 2 mesh sizes × 4 replicates) were used for the experiment.

In December 2016, the litterbags were immersed for 30 days in the Hermitage stream. After removal, the litterbags were immediately sealed in plastic bags to prevent the loss of litter material and were transported to the laboratory. The leaves were separated by species, which was possible even with small fragments of litter (owing to marked morphological differences among the species) and were carefully cleaned under water to remove macroinvertebrates and attached sediment particles.

Avoiding the central veins, six leaf discs (10 mm-diameter) for the single-species litters, or three disks for each species in the two-species litter mixtures, were cut for the purpose of microbial activity measurement. The remaining leaf material was frozen at -20 °C.

Microbial activity measurement

Microbial CO_2 production measurements, as a measure of overall heterotrophic microbial activity, were performed in order to evaluate the activity of the microbial communities that colonized the leaves after 30 days of decomposition. We adapted the protocol proposed by Anderson and Domsch (1973). Briefly, the six leaf disks taken in each litterbag were placed in 125 ml glass bottles with 25 ml of filtered water (GF/F glass microfiber filter, Whatman™) from the Hermitage stream and then preincubated for 12 h at 20 °C in the dark allowing the microbial respiration to saturate the water with CO_2. In the matter of fact, at constant temperature, it is only possible to increase the CO_2 concentration in the air phase of the respiration chamber if the aqueous phase is oversaturated in CO_2. After a night of remaining open to the atmosphere, the respiration chambers were hermetically sealed and the first sample of air was taken, the second air sample was sampled after a 4 h incubation at 20 °C in the dark. CO_2 production in a given time (i.e. 4 h) was then calculated as the difference between the final CO_2 concentration and the initial CO_2 concentration. Preliminary studies with our experimental conditions demonstrated that the CO_2 production was linear and, since all the process of incubation occurred in the dark, there was no photosynthesis and thus no alteration of the dissolved CO_2 partial pressure. In total, 440 respiration chambers, corresponding to the 440 litterbags, were prepared. At the beginning and at the end of the incubation period, 1 ml of air, taken with a syringe, was injected into a gas chromatograph (µGC SRA A 3000) in order to estimate the microbial activity (i.e. CO_2 air content). The leaf discs were then dried at 65 °C for 72 h and the CO_2 production was calculated as µg C-CO_2 per h and per g of litter dry mass (± 0.1 mg).

In parallel to the measurement of the microbial activity, the leaves remaining in the 440 litterbags were dried at 65 °C for 72 h, and then weighed to the nearest 0.1 mg. After weighing

224 the leaf disks and remaining leaf litter, all the litter material from a given litterbag was combined
225 and ground to a fine powder using a ball mill, before measuring the litter ash content. We
226 obtained ash-free dry mass by burning the combined sample at 550 °C for 4 h. Additional
227 samples were also used to estimate ash-free dry mass of the initial litter material. The percentage
228 of ash-free dry mass data was used to correct both the initial and the remaining leaf material
229 before the computation of (i) the percentage of litter mass loss after 30 days of field
230 decomposition and (ii) the microbial CO_2 production per h and per g of litter.
231
232 ***Litter trait measurement***
233 The initial litter traits were determined from four samples of each of the 10 litter species
234 (Supplementary Table S2). Prior to the chemical analysis, each litter sample was ground into
235 powder using a ball mill. The carbon (C), nitrogen (N), and sulfur (S) concentrations were
236 determined by thermal combustion, using a Vario Pyro cube CNS analyzer (Elementar France
237 SARL, Lyon, France). The phosphorus (P) concentration was measured colorimetrically using
238 the molybdenum blue method (Grimshaw and others 1989). To 80 mg of ground litter sample
239 8 ml of HNO_3 and 2 ml of H_2O_2 were added and the mixture was heated at 175 °C for 40 min
240 using microwaves (Ethos One, Milestone SRL, Italy). After this mineralization step, the sample
241 was diluted to a total of 50 ml. A hundred µl of sample, 100 µl of NaOH, 50 µl of mixed reagent
242 (emetic tartar and ammonium molybdate solution), and 50 µl of ascorbic acid were mixed
243 directly in a 96 well microplate. After 30 min at 40 °C, the reaction was completed, and the P
244 concentration was measured at 720 nm using a microplate reader (Victor, PerkinElmer,
245 Singapore). Following the mineralization step (i.e. the same as for P analysis), calcium (Ca),
246 magnesium (Mg), potassium (K), and sodium (Na) concentrations were measured using an
247 atomic absorption spectrometer (AAS, iCE 3000 series, ThermoScientific, China).

To assess the "mass-ratio hypothesis", we calculated the community-weighted mean (CWM) trait values of litter mixtures as the average trait values of litter mixtures following Garnier and others (2004) as: $\text{Trait}_{\text{CWM}} = \sum_{i=1}^{n} p_i \times trait_i$, where p_i is the relative abundance for species *i* in the litter mixture and $trait_i$ is the trait value for species *i*. These calculations were performed for each of the 8 litter traits. The highest scores of CWM were reached for litter mixtures containing two litter species with the highest nutrient concentrations (Fig. 1a). Since we predicted that both microbial activity and litter decomposition rate respond to the "mass-ratio hypothesis", we expected the increase in both decomposition rate and microbial activity to be positively correlated to the increase in CWM values.

To assess the "niche complementarity hypothesis", we calculated the functional dissimilarity (FD) of litter mixtures according to Rao's quadratic entropy (Botta Dukat 2005; Epps and others 2007) as: $\text{Trait}_{\text{FD}} = \sum_{i=1}^{n} \sum_{j=1}^{n} pipj * dij$, where p_i and p_j are the relative abundance for species *i* and *j* in the litter mixture, and d_{ij} the Euclidian distance between species *i* and *j* for the trait considered. These calculations were performed for each of the 8 litter traits. The highest scores of FD were reached for litter mixtures containing two species with very dissimilar nutrient concentrations (Fig. 1b). Since we predicted that the litter diversity effects respond to the "niche complementarity hypothesis", we expected the increase in relative litter mixture effects to be positively correlated to the increase in FD values.

Statistical analyses

All of the statistical analyses were conducted using R software (R Core Team 2013), with significance levels indicated as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.

A principal component analysis (PCA) was conducted using the values of the eight measured litter traits of the 10 tree species (Supplementary Table S2) in order to discriminate the five tree species with nutrient-rich litter (NRL) from the other five tree species with nutrient-

poor litter (NPL). The differences in the initial litter traits were assessed using one-way ANOVAs, followed by Tukey's tests to carry out post-hoc pairwise comparisons.

For the single-species litter, three-way ANOVAs, followed by Tukey's post hoc tests, were used to test for the effects of litter type (separated in NPL *vs.* NRL), litter species identity (10 litters), macroinvertebrate presence (fine-mesh bag [FMB] *vs.* coarse-mesh bag [CMB]), and their interactions on litter decomposition rate and microbial activity.

In order to further test whether litter decomposition and microbial activity differed between litter mixtures and single litter species, the relative litter mixture effects (RME) on litter decomposition rate and microbial activity were calculated. The RME was calculated as the relative difference between the observed litter decomposition rate/microbial activity (O) from the litter mixtures compared to those expected based on the respective single litter species treatments (E), following the formula $(O - E) / E \times 100\%$ (Wardle and others 1997). One-sample Student's *t*-tests were used to test whether the RME were significantly different from zero.

For the two-species litter mixtures, two-way ANOVAs, followed by Tukey's post hoc tests, were used to test for the effects of litter mixing (NPL-NPL, NPL-NRL and NRL-NRL), macroinvertebrate presence (FMB *vs.* CMB), and their interactions i) on litter decomposition rate and microbial activity and ii) on the RME on litter decomposition rate and on microbial activity.

For a more detailed understanding of how the mixture of leaf litter affected the litter decomposition rate and microbial activity, we evaluated the effects of the mean litter traits ($Trait_{CWM}$) and functional litter trait dissimilarities ($Trait_{FD}$) of the eight measured litter traits (Supplementary Table S2). First, a principal component analysis (PCA) was conducted using the CWM or the FD values of the eight measured litter traits across the 45 two-species litter mixtures. CWM1 and CWM2, and FD1 and FD2 represented the two first components of the

PCAs conducted using the CWM or the FD values across the litter mixtures, respectively. Second, multiple linear regression models were performed in order to decipher the relative contributions of $Trait_{CWM}$ and $Trait_{FD}$. In these models the effects of the $Traits_{CWM}$ (i.e. CWM1 and CWM2), $Traits_{FD}$ (i.e. FD1 and FD2), macroinvertebrate presence (FMB *vs.* CMB), and their interactions were tested i) on litter decomposition rate and microbial activity and ii) on the RME on litter decomposition rate and on microbial activity.

RESULTS

Litter traits

The PCA based on the element concentrations showed that the first PCA axis explained 50.2% of the variation and discriminated between the nutrient-rich litters (NRL) on the left and the nutrient-poor litters (NPL) on the right of the axis (Fig. 2).

Litter species incubated individually

The NRL exhibited two times more litter mass loss and microbial activity than the NPL (Table 1; Fig. 3). The presence of macroinvertebrates increased the litter mass loss and the microbial activity, but this effect was dependent on the litter type (litter type × mesh size interaction, Table 1). This significant interaction was explained by a stronger increase in litter mass loss and microbial activity in the NRL category (+59% litter mass loss and +33% microbial activity) compared to the NPL category (+23% litter mass loss and +28% microbial activity) (Fig. 3).

In addition, litter mass loss and microbial activity were significantly affected by litter species identity (Table 1). Within the NRL category, *Alnus* showed the highest litter mass loss compared to the four other species (Supplementary Fig. S1a), as well as a higher microbial

activity than *Carpinus*, *Acer*, and *Tilia* (Supplementary Fig. S1b). Within the NPL category, *Salix* and *Betula* showed both a higher litter mass loss and a higher microbial activity than the three other species (Supplementary Fig. S1a and S1b). The positive effect of the macroinvertebrate presence on the litter mass loss also varied according to the litter species identity (litter species × mesh size interaction, Table 1), as the effects ranged from +68% for *Alnus* to an absence of effect for *Fagus* (Supplementary Fig. S1a).

Effects of mixing low- and high-quality litter

Litter mass loss and microbial activity increased according to the gradient NPL-NPL < NPL-NRL < NRL-NRL mixtures (Table 2; Fig. 4a and 4c), with two times more litter mass loss and microbial activity in mixtures of two nutrient-rich litters compared to the mixtures of two nutrient-poor litters. The relative mixture effects (RME) on litter mass loss and on microbial activity showed a similar trend, with an increase in RME according to the same gradient (Table 2; Fig. 5). The NPL-NPL mixtures lost -3.5% litter mass compared with the expected values from the respective single litter species (Fig. 5a), while the observed microbial activity of the NPL-NPL mixtures did not differ significantly from the expected values (Fig. 5c). The NPL-NRL mixtures lost +5.3% litter mass than expected from the respective single litter species (Fig. 5a), while the observed microbial activity of the NPL-NRL mixtures did not differ from the expected values (Fig. 5c). The NRL-NRL mixtures showed +8.9% litter mass and +16.3% microbial activity than expected from the respective single litter species (Figs. 5a and 5c).

Litter mass loss and microbial activity were respectively 41% and 30% higher with macroinvertebrates than without (Table 2; Fig. 4b and 4d). The RME on litter mass loss and on microbial activity were also higher with macroinvertebrates than without (Table 2). Specifically, the litter mixtures exhibited synergistic effects in the presence of

348 macroinvertebrates, with +7.3% litter mass loss and +9.9% microbial activity than expected
349 from the respective single litter species (Figs. 5b and 5d).
350
351 ***CWM- versus FD-trait control over litter decomposition rate and microbial activity***
352 The PCA of the CWM traits showed that the first PCA axis (CWM1), explaining 50.2%
353 of the variation, was determined by high scores of P and Ca concentrations, and, to a lower
354 extent, by the scores of the K, Mg, Na, and S concentrations (Supplementary Fig. S2a). The
355 low scores of the second PCA axis (CWM2), explaining 21.9% of the variation, were related
356 to high values of N and C concentrations (Supplementary Fig. S2a). Regarding functional trait
357 dissimilarity, high scores of the first PCA axis (FD1), explaining 28.5% of the variation, were
358 related to the increase in dissimilarity in the Mg, S, and C concentrations, while the low scores
359 were related to the increase in dissimilarity in the N and Ca concentrations (Supplementary Fig.
360 S2b). The low scores along the second axis (FD2), explaining 22.0% of the variation, were
361 largely dependent on the increase in dissimilarity in the K, Na, and P concentrations
362 (Supplementary Fig. S2b).
363 When simultaneously evaluating the effects of the CWM and FD traits (Table 3), we
364 found that both litter mass loss and microbial activity were mainly controlled by the CWM
365 values (i.e. increasing mean nutrient concentrations within the litter mixture) compared to the
366 FD values (i.e. increasing dissimilarity in nutrient concentrations within the litter mixture). In
367 fact, CWM values (CWM1 + CWM2) explained respectively 9 and 15 times more of the overall
368 variance in litter mass loss and in microbial activity than FD values (FD1 + FD2) (Table 3).
369 When simultaneously evaluating the effects of the CWM and FD traits on RME, we
370 found that the RME on litter mass loss was controlled by the CWM (CWM1), the presence of
371 macroinvertebrates, and the interaction between the FD and the presence of macroinvertebrates
372 (FD2 × mesh size) (Table 3). Increasing CWM1 scores was related to higher RME (Fig. 6a).

The significant interaction between FD2 and mesh size (Table 3) showed that increasing FD in the initial K, Na, and P concentrations stimulated microbial-driven RME (Fig. 6d). Concerning the RME on microbial activity, it was found to be significantly affected only by CWM1 and the presence of macroinvertebrates (Table 3). Similar to what we observed for litter mass loss, increasing CWM1 scores was related to higher RME on microbial activity (Fig. 6e).

The RME on litter mass loss appeared to be more strongly correlated with the increase in P, Ca, Mg and Na concentrations than with the K, N, or S concentrations, and these relationships were more marked in the coarse-mesh litterbags in which macroinvertebrates were present, than in the fine-mesh litterbags in which macroinvertebrates were absent (Table 4). The RME on microbial activity appeared to be more strongly correlated with the increase in Ca, K, and Mg concentrations than in N and P concentrations, and these relationships were also more marked in the presence than in the absence of macroinvertebrates (Table 4).

DISCUSSION

Niche complementarity hypothesis vs. mass-ratio hypothesis for explaining litter diversity effects

We found evidence of leaf litter diversity effects on decomposition rates, including additive, synergistic, and antagonistic effects, as previously shown in other studies (Kominoski and others 2007; Srivastava and others 2009; Lecerf and others 2011; Handa and others 2014). Surprisingly, the pattern observed in our study highlighted that the largest mixture effects occurred in mixtures containing the combination of two nutrient-rich litters (NRLs), rather than in mixtures of one nutrient-rich litter (NRL) and one nutrient-poor litter (NPL), as predicted in our second hypothesis (Fig. 1b). Such a finding is in agreement with the mass-ratio hypothesis, and emphasizes the fact that increasing the mean value of the nutrient pool in litter mixtures

favors the occurrence of litter diversity effects. In any case our results pointed out that the litter diversity effects on decomposition were strongly litter-quality dependent. This finding is congruent with the recent study of Jabiol and Chauvet (2015) in which higher synergistic effects on litter decomposition occurred when *Alnus glutinosa* was combined with *Fraxinus angustifolia* (i.e. two NRLs) than when *Alnus glutinosa* was combined with *Quercus ilex* (i.e. one NRL and one NPL).

Surprisingly, $Trait_{CWM}$ proved to be a better predictor of litter diversity effects than $Trait_{FD}$, for both the coarse- and fine-mesh litterbags, contrasting with our second hypothesis. Indeed, based on the well-developed literature on the drivers of the litter decomposition process (e.g. Hättenschwiler and others 2005; Cornwell and others 2008; Gessner and others 2010; Makkonen and others 2012; Vos and others 2013; Handa and others 2014; Garcia-Palacios and others 2017), we expected that litter diversity effects occur when there are increases in the nutrient concentration dissimilarity of the litter mixtures (i.e. according to the niche complementary hypothesis, Fig. 1b), while the mean nutrient concentration within the litter mixture must only explain the litter decomposition rate (i.e. according to the mass-ratio hypothesis, Fig. 1a). The results from the coarse-mesh litterbags showed no evidence of nutrient dissimilarity effect ($Trait_{FD}$), conforming with the conclusion of Frainer and others (2015), which also highlighted, over a lower range of litter traits (N, P, and lignin), that no litter dissimilarity effect was involved in the litter diversity effects occurring in two-species mixtures. In contrast, $Trait_{FD}$ was linked to the litter diversity effects for the fine-mesh litterbags. In this case, litter mixtures with contrasting nutrient concentrations may have improved the availability of different nutrient sources for microorganisms (Schimel and Hättenschwiler 2007; Handa and others 2014), as suggested by the niche complementarity hypothesis. Filamentous fungi, including the aquatic hyphomycetes that dominate fungal communities on decomposing leaves in streams (Krauss and others 2011), can indeed extend their hyphae over considerable distances

423 in order to acquire remote resources that they transport to the locations of active hyphal growth
424 (Ritz 2006). It has previously been suggested that fungi-driven N transfer among litter species
425 varying in their initial N concentration may contribute to litter mixture effects (Schimel and
426 Hättenschwiler 2007; Vos and others 2013; Handa and others 2014). Thus, nutrients from a
427 nutrient-rich litter may be translocated to another litter depleted in nutrient (i.e. a nutrient-poor
428 litter; Schimel and Hattenschwiler 2007; Handa and others 2014). Therefore, our findings
429 highlighted that both $Trait_{CWM}$ and $Trait_{FD}$ contributed to explain litter diversity effects driven
430 by microorganisms alone. Interestingly, $Trait_{FD}$ did not explain litter diversity effects driven by
431 microorganisms and macroinvertebrates together. In this case, it could be hypothesized that the
432 additional nutrients provided by macroinvertebrate feces alleviated the nutrient limitation for
433 microorganisms (Wetzel 1995; Joyce and Wotton 2008), and consequently the $Trait_{FD}$
434 contribution to litter diversity effects.
435
436 ***Macroinvertebrates control over decomposition and litter diversity effects***
437 Across all of the 45 litter mixtures, litter mixture decomposition was not found to be
438 enhanced in the fine-mesh litterbags (i.e. additive effects), while the litter mixtures in the
439 coarse-mesh litterbags lost on average 7.3% more mass than the litter in the single-species
440 treatments (i.e. synergistic effects). The absence of litter mixture effects when
441 macroinvertebrates were excluded suggests that the activity of microbial decomposers alone
442 did not induce any effect of litter mixture on decomposition in the studied temperate stream.
443 Previous studies performed in lotic systems, where decomposition primarily involves
444 microorganisms, have also reported a lack of a synergism (Ferreira and others 2012; Bruder
445 and others 2014). For instance, similar to our findings, Jabiol and Chauvet (2015) reported that
446 no effect of litter mixture was observed when detritivores were excluded during the litter
447 decomposition in a Mediterranean stream in southern France. In a laboratory experiment, Swan

448 and Palmer (2006b) also reported that litter mixture effects were contingent on the feeding
449 activity of the isopod *Caecidotea communis*.

450 In homogenous species mixtures comprising litters of a single litter category,
451 macroinvertebrates have little choice to select among different qualities of leaves, as opposed
452 to the choice offered between leaf litter species in heterogeneous litter mixtures (Swan and
453 Palmer 2006b). However, as macroinvertebrates can simultaneously exploit multiple litter
454 species to meet their elemental demands (Leroy and Marks 2006), combining two NRLs that
455 nutritionally complement one another might stimulate the feeding activity of detritivores (Vos
456 and others 2013). For example, the mixing of one P-rich with one N-rich species in the present
457 study (such as *Acer platanoides* and *Alnus glutinosa*, respectively) increased litter mass loss of
458 the mixture by 14%, probably due to the increased nutritional value of such litter mixing. In
459 contrast, mixing two P-rich species (e.g. *Acer platanoides* and *Carpinus betulus*) increased litter
460 mass loss of the mixture by only 4%, while mixing two P-poor species (e.g. *Castanea sativa*
461 and *Fagus sylvatica*) decreased litter mass loss of the mixture by 10%. Alternatively in
462 NRL+NPL mixtures, high detritivore density may be promoted by the co-occurrence of a
463 suitable resource, such as that provided by the nutrient-rich litter, and a complex and structured
464 habitat provided by the nutrient-poor litter (Sanpera-Calbet and others 2009; Jabiol and others
465 2014).

466

467 ***Litter nutrients control over decomposition and litter diversity effects***

468 In agreement with our predictions and the literature, nutrient-rich litters decomposed
469 faster than nutrient-poor litters, confirming that nutrient concentration is an important
470 determinant of leaf litter decomposition (Kaspari and others 2008; Makkonen and others 2012;
471 Garcia-Palacios and others 2016). The rates of litter decomposition observed in the present
472 study were similar to those reported in other studies concerning temperate streams (e.g. Lecerf

and others 2007; Schindler and Gessner 2009; Frainer and others 2015). For instance, Lecerf and others (2007) reported 80% and 42% mass loss for *Alnus glutinosa* (i.e. a nutrient-rich litter) and *Quercus robur* (i.e. a nutrient-poor litter), respectively, after 34 days of decomposition in a temperate stream in central France. The results of the present study were also similar to the 16.5% mass loss for *Fagus sylvatica* (i.e. a nutrient-poor litter) after 30 days of decomposition obtained in the same Petit Hermitage stream in February 2005 (Piscart and others 2009).

Moreover, our results also highlighted the important role of nutrients not usually considered in litter diversity experiments. The first principal component of the CWM-trait PCA was the main driver of litter diversity effects in both fine- and coarse-mesh litterbags, and was driven by P, Ca, K, Mg, Na, and S. Their relative content in the leaves is closely related to one other (Garcia-Palacios and others 2016), hampering a straightforward interpretation of combination of elements. Nevertheless, when evaluating the individual contributions of litter nutrients, Ca and Mg appeared to be important drivers of litter diversity effects compared to N (and to P only for microbial activity), suggesting for the first time that these two nutrients may play an important role in litter diversity effects in addition to, or independently of, the P or N content. The Ca content is known to positively affect the growth and activity of aquatic hyphomycetes, by enhancing the fungal capacity to transfer N between distinct litter types (Jenkins and Suberkropp 1995). Meanwhile, Ca and Mg are known to be essential elements in the diet of macroinvertebrates, since they are required for enzymatic reactions, nerve connections, muscle function, and skeleton formation (National Research Council 2005). Moreover, in some temperate streams, crustaceans could represent up to 95% of shredder biomass (Piscart and others 2011), and the Ca content of leaves could be a significant source of Ca for their cuticles (Cairns and Yan 2009), especially in streams with a low Ca content in the water (Glazier 1998), such as in Brittany, where the present study was conducted.

CONCLUSION

As expected, increasing mean nutrient concentration and macroinvertebrate presence favored both microbial activity and litter decomposition rate. In addition, we experimentally demonstrated for the first time that the mass-ratio hypothesis (i.e. $Trait_{CWM}$) overrules the niche complementarity hypothesis ($Trait_{FD}$) as a driver of litter diversity effects in a temperate stream. In fact, in strong contrast to our expectations, the combination of two nutrient-rich litters yielded the highest litter mixture effects. In addition, the synergistic effects of litter mixing were mainly evident in the presence of macroinvertebrates. Both community weighted mean traits ($Trait_{CWM}$) and the functional dissimilarity of litter traits ($Trait_{FD}$) contributed to explain the litter mixture effects. There was no support for $Trait_{FD}$ explaining litter diversity effects in the presence of macroinvertebrates, while both $Trait_{CWM}$ and $Trait_{FD}$ contributed to explain litter mixture effects driven by microorganisms alone. Finally, Ca and Mg, which are often neglected in decomposition studies in streams, were found to be important drivers of litter mixture effects.

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AUTHORS' CONTRIBUTIONS

M.S., H.R.P., N.L.B. and C.P. conceived and performed the experiments. M.S. analyzed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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TABLES

Table 1. Effects of litter quality (NPL *vs.* NRL), litter species identity (10 species), litterbag mesh size (fine-mesh *vs.* coarse-mesh litterbags), and their interactions on litter decomposition rate and microbial activity in the single-species litterbags. d.f. = degrees of freedom, %SS = percentage of type III sums of squares. *F*-values and associated *P*-values (with the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$) are indicated.

		Litter decomposition		Microbial activity	
	d.f.	%SS	*F*-value	%SS	*F*-value
Litter quality (LQ)	1	59.1	854.8***	60.0	239.7***
Litter species (LS)	8	12.6	22.8***	11.3	5.6***
Mesh size (MS)	1	15.8	228.9***	11.1	44.3***
LQ × MS	1	6.6	95.9***	1.4	5.7*
LS × MS	8	1.6	3.0**	1.1	0.6
Residuals	60	4.2		15.0	

753 **Table 2.** Effects of litter quality mixing (NPL-NPL, NPL-NRL and NRL-NRL), litterbag mesh
754 size (fine-mesh *vs.* coarse-mesh litterbags), and their interactions i) on litter decomposition rate
755 and microbial activity and ii) on relative mixture effects (RME) on litter decomposition rate
756 (LD) and on microbial activity (MA). d.f. = degrees of freedom, %SS = percentage of type III
757 sums of squares. *F*-values and associated *P*-values (with the respective symbols * for $P < 0.05$,
758 ** for $P < 0.01$, and *** for $P < 0.001$) are indicated.
759

		Litter decomposition		Microbial activity		RME on LD		RME on MA	
	d.f.	%SS	*F*-value	%SS	*F*-value	%SS	*F*-value	%SS	*F*-value
Litter quality (LQ)	2	49.1	617.5 ***	55.5	506.2 ***	21.6	58.0 ***	19.7	51.9 ***
Mesh size (MS)	1	30.8	776.2 ***	23.1	421.8 ***	11.5	61.5 ***	13.4	70.7 ***
LQ × MS	2	6.0	75.3 ***	2.1	18.6 ***	0.8	2.3	0.0	0.1
Residuals	354	14.1		19.3		66.0		66.8	

760
761

Table 3. Effects of community-weighted mean traits (CWM), functional trait dissimilarities (FD), litterbag mesh size (fine-mesh *vs.* coarse-mesh litterbags), and their interactions i) on litter decomposition rate and microbial activity and ii) on relative mixture effects (RME) on litter decomposition (LD) and on microbial activity (MA). CWM1 and CWM2, and FD1 and FD2 represented the two first components of the PCAs conducted using the CWM or the FD values in Supplementary Fig. S2. d.f. = degrees of freedom, %SS = percentage of type III sums of squares. *F*-values and associated *P*-values (with the respective symbols * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$) are indicated.

		Litter decomposition		Microbial activity		RME on LD		RME on MA	
	d.f.	%SS	F-value	%SS	F-value	%SS	F-value	%SS	F-value
CWM1	1	48.8	2693.2 ***	55.1	1344.1 ***	15.4	81.8 ***	23.1	129.3 ***
CWM2	1	1.6	86.4 ***	1.6	38.1 ***	0.3	1.4	0.3	1.7
FD1	1	3.7	202.3 ***	1.8	43.2 ***	0.2	0.8	0.2	1.1
FD2	1	1.9	107.1 ***	1.4	33.9 ***	3.7	19.9 ***	0.5	2.9
Mesh size (MS)	1	30.8	1704.0 ***	22.9	559.5 ***	11.5	60.8 ***	13.3	74.6 ***
CWM1 × MS	1	6.0	332.0 ***	1.9	46.5 ***	0.3	1.3	0.0	0.3
CWM2 × MS	1	0.1	3.4	0.3	7.1 **	0.7	3.6	0.1	0.3
FD1 × MS	1	0.7	40.1 ***	0.7	17.5 ***	0.4	2.0	0.2	1.2
FD2 × MS	1	0.1	5.3 *	0.1	2.7	1.6	8.3 **	0.0	0.2
Residuals	350	6.3		14.3		66.0		62.2	

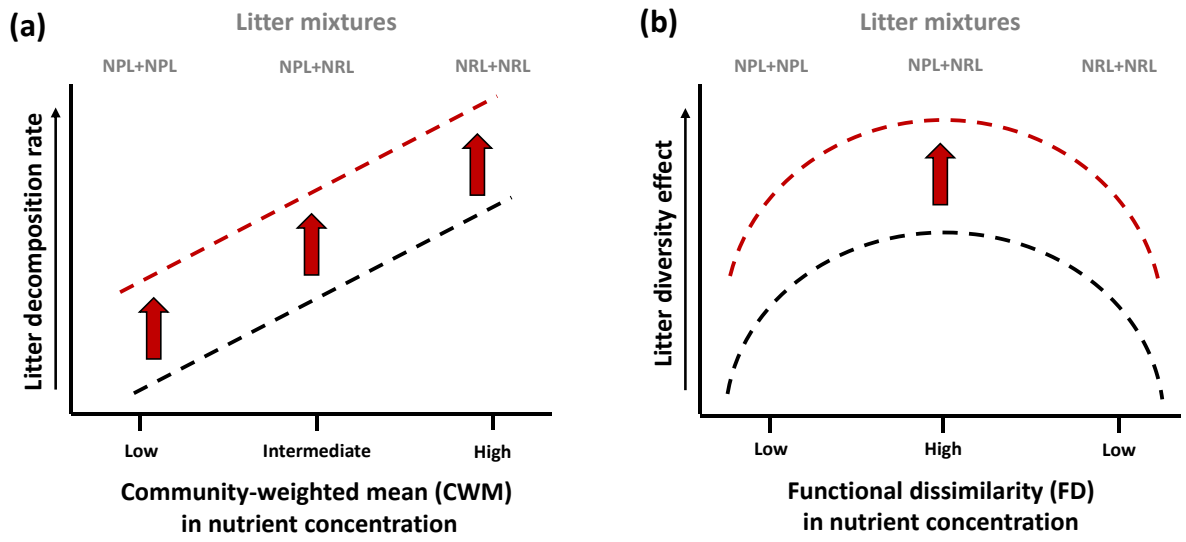
773 **Table 4.** Relationships between CWM values of chemical element concentrations and relative
774 mixture effects (RME) on litter decomposition rate and on microbial activity in fine-mesh and
775 coarse-mesh litterbags. Adjusted R^2 and associated *P*-values (* $P < 0.05$, ** $P < 0.01$, *** $P <$
776 0.001) are indicated.
777

	C	N	P	Ca	K	Mg	Na	S
RME on litter decomposition								
Fine-mesh litterbags	0.19 **	0.19 **	0.28 ***	0.26 ***	0.14 **	0.21 **	0.18 **	0.09 *
Coarse-mesh litterbags	0.17 **	0.12 *	0.27 ***	0.35 ***	0.24 ***	0.30 ***	0.33 ***	0.15 **
RME on microbial activity								
Fine-mesh litterbags	0.12 *	0.02	0.13 *	0.27 **	0.26 ***	0.31 ***	0.27 ***	0.21 ***
Coarse-mesh litterbags	0.07	0.16 **	0.27 ***	0.41 ***	0.30 ***	0.43 ***	0.16 **	0.16 **

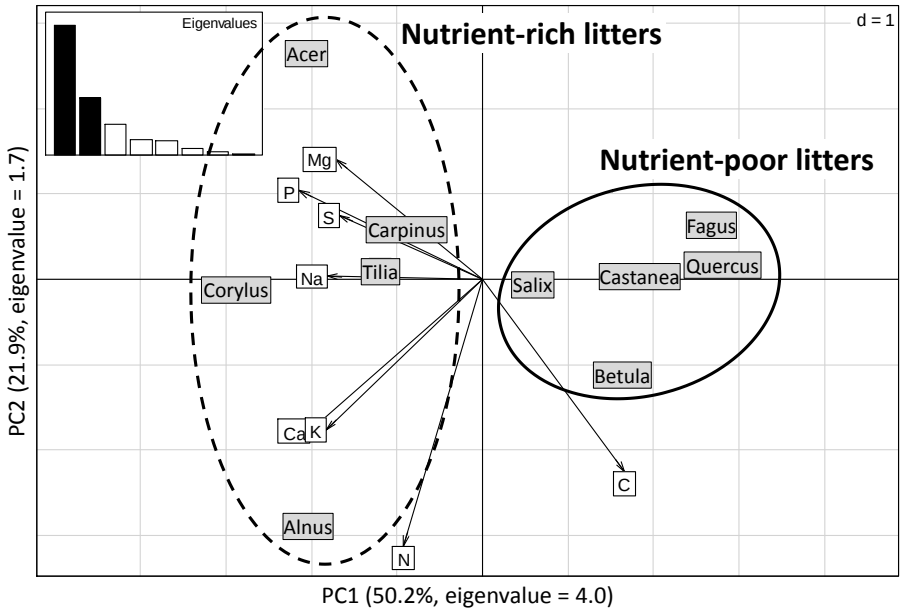
778
779

FIGURES

Fig. 1. (a) Hypothetical relationship between the different categories of litter mixtures and the litter decomposition rate of these litter mixtures. According to the mass-ratio hypothesis, the decomposition rate increases with the increase in CWM values in nutrient concentration within a litter mixture. (b) Hypothetical relationship between the different categories of litter mixtures and the intensity of litter diversity effects occurring in these litter mixtures. According to the niche complementary hypothesis, the intensity of litter diversity effects increases with the increase in FD values in nutrient concentration, which would be shown by an increase in litter decomposition rate in the present study. More precisely, this increase in decomposition rate, due to synergistic effects between the two litters in mixtures, will be higher in litter mixtures exhibiting intermediate nutrient concentration compared to litter mixtures exhibiting low or high nutrient concentrations (Fig. 1a). NPL = nutrient-poor litter, NRL = nutrient-rich litter. For both relationships, red dotted lines illustrate hypothetical increases in both litter decomposition rate and litter diversity effects mediated by macroinvertebrate presence.



798 **Fig. 2.** Principal component analysis (PCA) based on the chemical element concentrations of
799 the 10 litter species. Variance explained by each principal component and associated
800 eigenvalues are shown in brackets.
801



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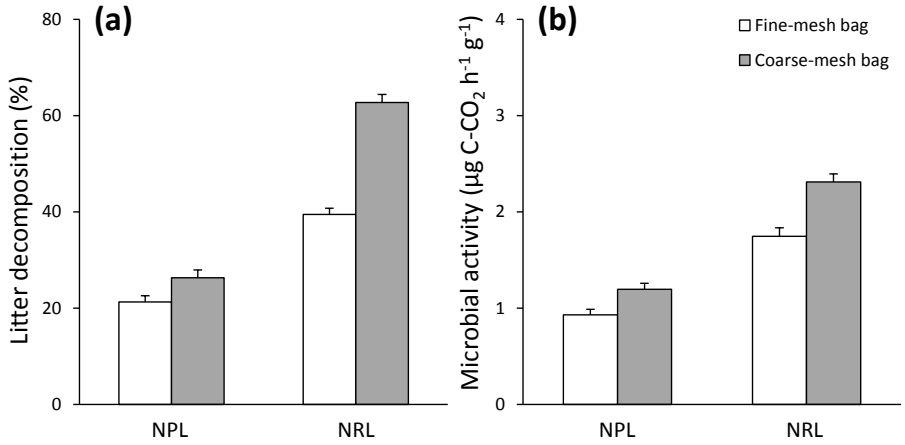
803

804 **Fig. 3.** Litter decomposition rate (a) and microbial activity (b) in the single-species litterbags
805 according to litter quality (nutrient-poor litter [NPL] *vs.* nutrient-rich litter [NRL]) and to
806 litterbag mesh size (fine-mesh *vs.* coarse mesh litterbags). Each bar represents the mean value
807 ± SE; n= 20. The litter decomposition rate is indicated in percent mass loss relative to the initial
808 mass. The microbial activity is expressed as µg C-CO_2 production per h and per g of litter.
809



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811

Fig. 4. Litter decomposition rate (a, b) and microbial activity (c, d) according to litter quality mixing (a, c) and to litterbag mesh size (b, d). Each bar represents the mean value ± SE. NPL = nutrient-poor litter, NRL = nutrient-rich litter, FMB = fine-mesh litterbag, CMB = coarse-mesh litterbag. Different letters denote significant differences between treatments with a < b < c.

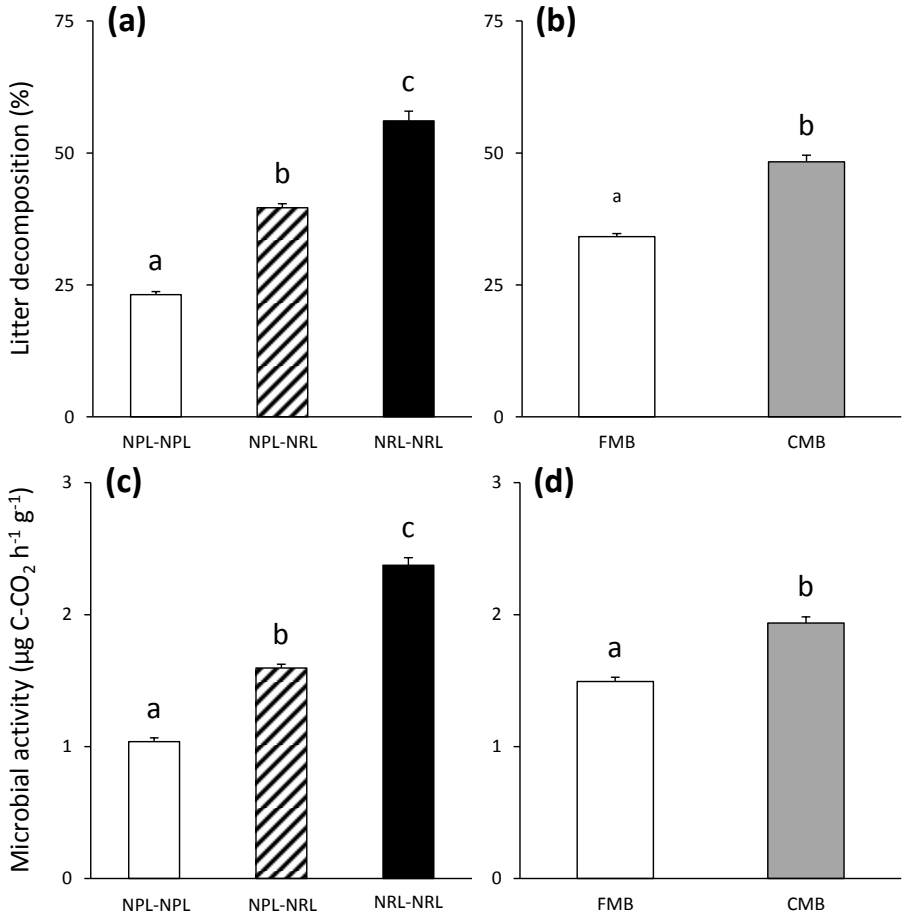

Fig. 5. Relative mixture effects (RME) on litter decomposition rate (a, b) and on microbial activity (c, d) according to litter quality mixing (a, c) and to litterbag mesh size (b, d). Each bar represents the mean value ± SE. NPL = nutrient-poor litter, NRL = nutrient-rich litter, FMB = fine-mesh litterbag, CMB = coarse-mesh litterbag. The RME on litter decomposition rate and on microbial activity are indicated as the relative difference between the observed and the expected values from the respective single litter species treatments. Different letters denote significant differences between treatments with a < b < c.

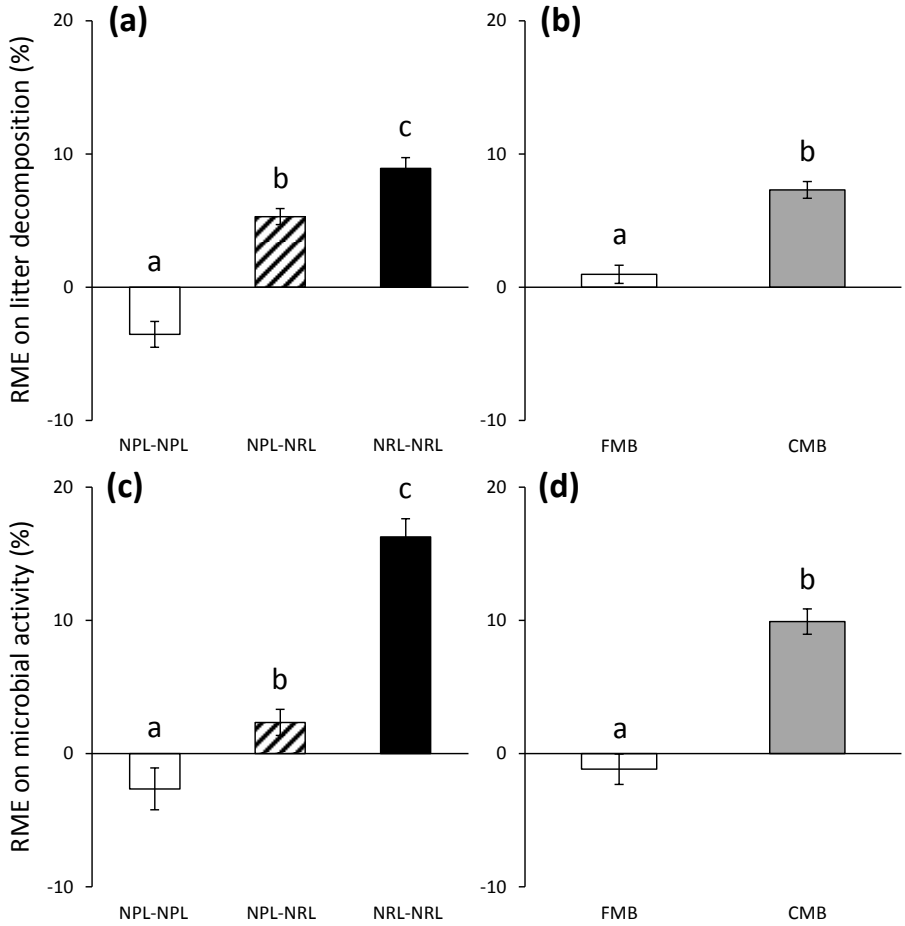

Fig. 6. Relative mixture effects (RME) on litter decomposition rate (panels a, b, c and d) and on microbial activity (panels e, f, g and h) as a function of community weighted mean traits (CWM1 and CWM2 from the PCA using the CWM values, Supplementary Fig. S2a) and functional trait dissimilarities (FD1 and FD2 from the PCA using the FD values, Supplementary Fig. S2b) in fine-mesh (white symbol) and coarse-mesh (grey symbol) litterbags. The RME on litter decomposition rate and on microbial activity are indicated as the relative difference between the observed and the expected values from the respective single litter species treatments. Significant relationships are indicated with dotted (fine-mesh litterbags) or grey (coarse-mesh litterbags) lines.