

# Stream nitrogen concentration, but not plant N-fixing capacity, modulates litter diversity effects on decomposition

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## Summary

1. We are facing major biodiversity loss and there is evidence that such loss can alter ecosystem functioning. However, the effects of plant diversity on decomposition – a key component of the global carbon cycle – are still unclear. A recent study suggested that a plant trait – their nitrogen (N)-fixing capacity – could mediate effects of litter diversity on decomposition by means of a microbial transfer of N from N-fixers to non-fixers.
2. We explored this possibility in a microcosm experiment in which we manipulated litter species richness (one, two or four species), N-fixing capacity (N-fixer or non-fixer species), the presence of detritivores (*Sericostoma pyrenaicum* larvae present or absent) and water N concentration [natural stream water (0.366 mg L<sup>-1</sup> of NO<sub>3</sub>-N) or elevated N concentration (five times the natural concentration: 1.835 mg L<sup>-1</sup>)].
3. We show that litter diversity accelerated decomposition by micro-organisms and detritivores (by 7% and 15% respectively), mostly through complementarity effects. However, enhanced decomposition did not result in higher detritivore growth, possibly because all litter combinations provided sufficient resources for their maximum growth.
4. The plant N-fixing capacity had no effect on decomposition, which varied among species most likely because of differences in a combination of litter traits. Detritivores maximized the consumption of their preferred resource in litter mixtures, but also exploited less preferred resources, and their C : N ratios increased during the experiment regardless of litter type or water N concentration.
5. Microbial decomposition of litter with low N content was enhanced at elevated water N concentration, suggesting that micro-organisms used nutrients from the water when those nutrients were limiting in leaf litter. In contrast, detritivore growth was impaired at elevated water N concentration, possibly because a stoichiometric imbalance entails metabolic costs.
6. Our findings suggest that loss of plant diversity in riparian forests would mostly affect decomposition in streams of high nutrient status, where effects on microbial decomposition would be more evident and detritivore populations may be reduced.

**Key-words:** decomposition rate, detritivores, functional traits, litter breakdown, nitrogen-fixing plants, species richness

## Introduction

The current major rate of biodiversity loss (Barnosky *et al.* 2011), and its potential consequences for ecosystem

functioning, goods and services (Cardinale *et al.* 2012), have motivated hundreds of experimental studies testing how changes in species richness might alter rates of primary production and plant litter decomposition (Schmid *et al.* 2009; Cardinale *et al.* 2011). Relevant studies on primary production have typically demonstrated that a reduction in species richness decreases the efficiency with which

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biological communities capture resources and convert them into new plant biomass, the mechanisms for which are well understood (Hector *et al.* 2009). In contrast, our understanding of how species loss affects plant litter decomposition is still in its infancy (Cardinale *et al.* 2011), despite the importance of this process. Plant litter decomposition is a key component of the global carbon (C) cycle, as 90% of terrestrial plant biomass produced each year dies and is stored or decomposed in soils and fresh waters, with major consequences for nutrient cycling and carbon dioxide emission rates (Gessner *et al.* 2010; Raymond *et al.* 2013).

Experimental studies have failed to show a clear effect of plant species richness on decomposition rates. Two meta-analyses, including 90 and 84 observations, respectively, found either no effects of richness on decomposition rates (Srivastava *et al.* 2009), or a significant but small effect (litter mixtures lost 5% more mass than the average monoculture) (Cardinale *et al.* 2011). Subsequent studies have similarly found a lack of clear effects, and demonstrated that species identity in litter mixtures, rather than species richness *per se*, is the major influence on decomposition rates (Ferreira, Encalada & Graça 2012; Boyero *et al.* 2014; Bruder *et al.* 2014).

The lack of a clear, unidirectional effect of plant species richness on decomposition rate could be related to the wide variety of functional traits contained in different litter mixtures. A recent study showed that mixing litter with different key traits (acquisition strategies for C and N, and litter recalcitrance) resulted in accelerated C and N loss compared to monocultures, and the pattern was consistent across biomes and ecosystem types (Handa *et al.* 2014). Specifically, litter diversity effects on C and N loss were largely explained by the interaction between N-fixing plants [which have symbiotic bacteria that fix atmospheric N and make it available to the plant (Franché, Lindström & Elmerich 2008)] and non-N-fixing plants, which were deciduous and rapid decomposers. These results suggested that N could be transferred from litter of N-fixers to that of non-fixers, possibly through fungal decomposers, which may use the N reservoir of litter from N-fixers and boost the use of high-quality C from litter of non-fixers.

Here we explore the effects of mixing litter from N-fixer and non-fixer plants (hereafter N-fixer and non-fixer litter respectively) on decomposition rates in a laboratory experiment. We mixed litter from different species of these two functional types and compared their decomposition rates with those of their monocultures in the presence and absence of detritivores. We also manipulated the concentration of inorganic N in the water to investigate whether it affected any interaction between N-fixer and non-fixer litter. We predicted that (i) an increase in litter species richness would promote decomposition due to positive complementarity effects (Boyer, Kertesz & Bruno 2000), and would enhance detritivore growth through the use of a greater variety of litter types by detritivores (i.e. a balanced diet effect; DeMott 1998); (ii) decomposition of N-fixer

litter would be faster than that of non-fixer litter, because the higher N content of N-fixer litter promotes the activity of microbial decomposers and detritivores; because of this, detritivore growth would be higher on N-fixer than non-fixer litter; (iii) decomposition and detritivore growth would be enhanced in litter mixtures containing both N-fixers and non-fixers, compared to mixtures of a single functional type or to monocultures, because the high N content of N-fixer litter would boost the use of C from non-fixer litter, resulting in a more efficient use of both resources (as suggested in Handa *et al.* 2014) and (iv) any effects of litter type on decomposition would only occur when N is limiting in the water; when N is not limiting, microbial decomposers would be able to use it (Cheever *et al.* 2013), and the N contained in N-fixer litter would be superfluous.

## Materials and methods

### PLANT SPECIES AND FUNCTIONAL TYPES

In Europe, N-fixing plants include several common riparian tree species such as the black alder *Alnus glutinosa* [L.] Gaertn. (Betulaceae) and the exotic black locust *Robinia pseudoacacia* L. (Fabaceae) (hereafter *Alnus* and *Robinia*). Both species are known to greatly increase the N content of soils (Von Holle *et al.* 2005), and their leaves generally show higher N content than other common riparian species (Alonso, González-Muñoz & Castro-Díez 2010; Casas *et al.* 2013). We used these two species in our experiment, together with two other common riparian species that are not associated with N-fixing bacteria: the black poplar *Populus nigra* L. (Salicaceae) and the grey willow *Salix atrocinerea* Brot. (Salicaceae) (hereafter *Populus* and *Salix*). Litter of these two species generally has low N content (Casas & Gessner 1999), but is similar to the other selected species in terms of C allocation strategies (i.e., they are all deciduous) and recalcitrance [i.e. they all have relatively fast decomposition rates, although *Alnus* decomposes at a faster rate than the other three species (Casas & Gessner 1999; Alonso, González-Muñoz & Castro-Díez 2010; Pozo *et al.* 2011) and has lower lignin content (c. 12% dry mass for *Alnus*, 15% for *Robinia*, 18% for *Salix* and 23% for *Populus*) (Chauvet 1987; Gallardo & Merino 1992; Alonso, González-Muñoz & Castro-Díez 2010)]. The four species selected were among the most common riparian species in the study area.

Freshly fallen leaves were collected from the ground at various locations from the Biscay province, northern Spain (43.22°N 3.27°W; 43.33°N 2.97°W; 43.29°N 2.99°W), in November 2014. In the laboratory, discs of 12-mm diameter were cut from the leaves using a cork borer. As we could not avoid the central nerve when cutting the discs in *Robinia* leaflets (which are <3 cm wide), we included the nerves in discs of all species, but avoided the widest part next to the petiole. Discs were air-dried and weighed in groups of 10, 20 or 40, to be used in the different experimental treatments.

### LEAF QUALITY

We determined the initial leaf quality of each plant species (N and P contents, C : N and N : P ratios and ash content) to examine its possible influence on our results. Five replicates of 20 discs per species were air-dried and ground into powder (1-mm screen) and their initial nutrient contents determined. C and N contents [% of total dry mass (DM)] were determined using a Perkin Elmer series

II CHNS/O elemental analyser (Perkin Elmer, Norwalk, CT, USA), and P content (%) was measured spectrophotometrically after autoclave-assisted extraction (APHA 1998). Five discs per species were oven dried (60 °C, 72 h) to determine their DM and then incinerated (550 °C, 4 h) to determine their ash-free dry mass (AFDM) and calculate ash content (%). We explored differences in leaf quality (N and P content, C : N and N : P ratios and ash content) with linear models followed by multiple comparisons.

#### EXPERIMENTAL SET UP

In May–June 2015 we conducted an experiment in 220 microcosms (8 cm-diameter glass cups) within a controlled-temperature room set at 10 °C, which was lower than the average temperature of streams when detritivores were collected (c. 13 °C) but which significantly reduced evaporation. Each microcosm contained 40 leaf discs that belonged to one species (monocultures) or to two or four species (litter mixtures of all possible species combinations, containing 20 or 10 discs per species respectively; Fig. 1). Leaf discs of the same species were marked and kept together in 10-disc groups using labelled safety pins, so they could be easily identified at the end of the experiment. For each plant treatment, 10 replicate microcosms included detritivores and 10 did not. Each replicate with detritivores contained three larvae of the caddisfly *Sericostoma pyrenaicum* Pictet, 1865 (Sericostomatidae), which is a common detritivore in the study area. Detritivore biomass per microcosm was on average 28.07 mg ( $\pm 5.48$  SD; Table S3, Supporting Information) [i.e. the average individual biomass was c. 9.4 mg, which corresponds to the last (7th) larval instar in this species (Basaguren, Riaño & Pozo 2002)] and did not differ between plant species richness, plant functional type or water N concentration treatments ( $P > 0.27$  in all cases; Table S4). Larvae were collected from leaf litter in streams of the Agüera watershed and starved for 48 h prior to the experiment. For each plant/detritivore combination, half of the microcosms contained 250 mL of filtered (100  $\mu$ m) stream water (mean  $\pm$  SE of  $\text{NO}_3\text{-N}$  concentration =  $0.366 \pm 0.010$  mg  $\text{L}^{-1}$ ) and the other half contained 250 mL of filtered stream water with added potassium nitrate to elevate N concentration to five times the natural concentration (i.e., to  $1.835 \pm 0.031$  mg  $\text{L}^{-1}$ ), which is similar to the highest concentration found in the study area (Barba *et al.* 2010). Concentration of soluble reactive phosphorus was  $9.5 \mu\text{g L}^{-1}$ . We added fine sand and pebbles (previously incinerated at 550 °C for 4 h and washed to remove ash) to each microcosm to provide environmental heterogeneity and material for caddisfly case construction.

The experiment was run for 24 days. Initially, only the leaf discs were added to the microcosms to allow initial conditioning and leaching of soluble compounds. On day 3 we replaced the

water and added the detritivores. Water was again replaced on days 11 and 18, and the experiment was terminated on day 24. Microcosms were monitored every 2 days to ensure there was leaf material of every species available during the experimental period. At the end of the experiment, all leaf material was collected (fragments were identified based on colour and morphology), oven dried (60 °C, 72 h) to determine DM, and then incinerated (550 °C, 4 h) to determine AFDM. DM and AFDM showed a very strong relationship ( $r^2 = 0.99$ ,  $F_{1,219} = 20055.2$ ,  $P < 0.001$ ), so only AFDM was used in the analyses. We used five additional sets of 40 leaf discs per species to calculate a DM/AFDM correction factor, which was used to estimate initial AFDM of each microcosm. Leaf mass loss due to leaching was not measured during the experiment, but we measured it a posteriori (several months later) on five additional sets of 40 leaf discs per species, which were submerged in filtered stream water for 3 days, oven-dried and weighed.

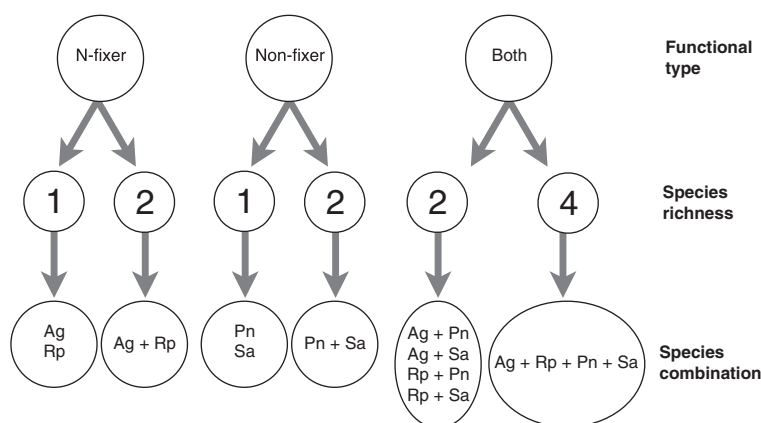
Detritivores were oven dried (60 °C, 72 h) to determine their final DM; initial DM was estimated from a case length (CL)/DM relationship, calculated using 26 additional individuals of similar case length to those used in the experiment ( $\text{DM} = 0.17 \times \text{CL}^2 - 2.87 \times \text{CL} + 14.15$ ;  $r^2 = 0.96$ ). Detritivores were ground and analysed in a Perkin Elmer series II CHNS/O elemental analyser (Perkin Elmer) to determine their C : N ratio; the initial C : N ratio was determined using five replicates of three individuals from the pool of 26 additional individuals used to estimate initial DM.

#### RESPONSE VARIABLES

Our experiment allowed us to examine the influence of plant species richness, plant functional type (in terms of N-fixing capacity), detritivores (presence and biomass) and water N concentration on litter decomposition rate and detritivore growth. Decomposition rate was estimated through the relative litter mass loss (LML) during the experiment:  $\text{LML} = (\text{initial AFDM} - \text{final AFDM}) / \text{initial AFDM}$ . We calculated LML separately for each plant species in a microcosm, and total LML of all component species in a microcosm. Because the leaf material used in the leaching trial had been stored in the laboratory for several months, apparently increasing leaching (Fig. S1), we did not use LML resulting from this leaching trial to correct initial leaf mass in the experiment, but used the leaching data for comparative purposes among species. We quantified detritivore growth (DG) as the relative growth during the experiment:  $\text{DG} = (\text{final DM} - \text{initial DM}) / \text{initial DM}$ .

As discs of different plant species were weighed separately, we could also explore the potential mechanisms responsible for any effect of species mixtures on decomposition. We used the additive partitioning method (Loreau & Hector 2001) to measure the *Net*

**Fig. 1.** Experimental design with different litter functional types (N-fixer, non-fixer or both), species richness levels (one, two or four species) and species combinations (Ag, *Alnus glutinosa*; Rp, *Robinia pseudoacacia*; Pn, *Populus nigra*; Sa, *Salix atrocinerea*).



Effect of diversity on decomposition, as well as the relative contribution of a *Complementarity Effect*, which can occur through resource partitioning or from synergistic or antagonistic interactions, and a *Selection Effect*, which arises when the presence of a particular species with high (or low) decomposition rate dominates the rate of decomposition of a mixture (Loreau & Hector 2001; Handa *et al.* 2014). The net effect was calculated as the difference between the observed LML of a mixture and its expected LML, which was based on LML in the monocultures ( $\Delta\text{LML} = \text{LML}_O - \text{LML}_E$ ). The complementarity effect was calculated as the average deviation from expected LML of species in a mixture multiplied by the mean LML of species in monoculture and the number of species in the mixture (mean  $\Delta\text{LML} \times \text{mean LML} \times N$ ). The selection effect was calculated as the covariance between LML of species in monoculture and their  $\Delta\text{LML}$  multiplied by the number of species [ $\text{cov}(\Delta\text{LML}, \text{LML}) \times N$ ].

## STATISTICAL ANALYSES

We used linear models and a backward model selection procedure based on the Akaike Information Criterion (AIC) to explore variation in leaf mass loss (LML), net diversity, complementarity and selection effects and detritivore growth (DG) and C : N ratios, in relation to plant species richness (one, two and four for LML, DG and C : N ratios; two and four for the other variables, as diversity effects are calculated by comparing species mixtures with the monocultures), plant functional type (N-fixer, non-fixer or both), detritivore presence, water treatment (natural or N addition), and the interactions among these factors. Initial data exploration using Cleveland dot- and boxplots revealed no outliers in the data, so there was no need for transformations (Zuur & Ieno 2015). However, data exploration showed clear differences in the variance of each response variable between detritivore treatments (Fig. S2). For this reason, and to avoid very complex models with many interactions, we examined each of these treatments separately and used a separate model to explore variation in each variable (except DG) between detritivore treatments.

Multi-panel boxplots for each response variable vs. species richness and functional type showed that the homogeneity of variances assumption for linear models was violated, requiring the use of a variance structure that takes these differences into account [VarIdent function of 'nlme' R package (Pinheiro *et al.* 2016) in R software (version 3.2.2; R Core Team 2015)]. Detritivore biomass (final DM) was included in the model for microcosms with detritivores, to account for the higher mass loss most likely caused by larger detritivores (Boyero *et al.* 2014). All variables were treated as categorical except detritivore biomass, which was continuous.

The models were fitted using the gls function (generalized least squares) and restricted maximum likelihood (REML) method in the 'nlme' R package. The optimal variance structure was defined by comparing models with different variance structure (using VarIdent), and evaluated using AIC. The optimal models allowed residual spread to vary in relation to each species combination (LML and DG), each species and water treatment combination

(net diversity, complementarity and selection effects), each species and detritivore presence combination (LML comparing detritivore treatments), or detritivore presence (net diversity, complementarity and selection effects comparing detritivore treatments). Visual exploration of residuals indicated no violation of linear model assumptions. Pairwise multiple comparisons were addressed with Tukey tests using the glht function of the 'multcomp' R package (Hothorn, Bretz & Westfall 2008).

We further explored whether results for LML depended on plant species identity in a mixture, using LML data for each plant species. We followed the same steps as above to define the optimal random and fixed structure of models. For these models, we also tested the autocorrelation between species in the same replicate (ID variable), because their LMLs were not independent of each other. Autocorrelation was evaluated with the acf function in R, and comparing model improvement with AIC (Zuur *et al.* 2009). Autocorrelation occurred only when detritivores were present, and was removed by adding a correlation structure to the model (corCompSymm function also in the 'nlme' R package).

## Results

### LEAF QUALITY

Leaf quality differed among plant species (Table 1): N content was highest for *Alnus* and lowest for *Populus*; P content was highest for *Alnus* and lowest for *Robinia*; the C : N ratio was highest for *Populus* and lowest for *Alnus*; the N : P ratio was highest for *Robinia* and lowest for *Populus* and ash content was highest for *Populus* and lowest for *Alnus*.

### LEAF MASS LOSS

Microcosms had leaf litter present throughout the experiment, except that at the end of the experiment *Alnus* litter was absent from 5% of the microcosms containing this species (Fig. S3). Leaf mass loss (LML) was, on average, more than twice as high when detritivores were present (54%) as when they were absent (25%) ( $F_{1,218} = 529.4$ ,  $P < 0.001$ ). On average, the contribution of detritivores to LML was 68% ( $\pm 0.02$  SD) and varied from 31 to 89% (Table S5).

When detritivores were present, LML was affected by plant species richness, plant functional type and water N concentration (Tables 2, S1): LML was greater in microcosms having two vs. one, four vs. one and four vs. two species (Fig. 2a); it was higher for N-fixers or for both functional types together than for non-fixers (Fig. 2c) and

**Table 1.** Mean ( $\pm$ SE) of nitrogen (N) and phosphorus (P) content (% dry mass), C : N and N : P ratios and ash content (% dry mass), for each leaf species based on measurements of five replicates. Different letters indicate significant differences on the basis of a linear model followed by pairwise multiple comparisons (significant values  $P < 0.05$ )

Species	N	P	C : N	N : P	Ash
<i>Alnus glutinosa</i>	2.9 $\pm$ 0.1 <sup>a</sup>	0.10 $\pm$ 0.001 <sup>a</sup>	19.8 $\pm$ 0.2 <sup>c</sup>	62.7 $\pm$ 1.3 <sup>b</sup>	4.59 $\pm$ 1.29 <sup>d</sup>
<i>Robinia pseudoacacia</i>	1.5 $\pm$ 0.03 <sup>b</sup>	0.04 $\pm$ 0.002 <sup>c</sup>	35.2 $\pm$ 0.5 <sup>b</sup>	90.8 $\pm$ 6.6 <sup>a</sup>	13.33 $\pm$ 2.73 <sup>b</sup>
<i>Populus nigra</i>	0.7 $\pm$ 0.03 <sup>c</sup>	0.08 $\pm$ 0.001 <sup>b</sup>	67.4 $\pm$ 2.2 <sup>a</sup>	20.2 $\pm$ 1.0 <sup>d</sup>	15.49 $\pm$ 1.31 <sup>a</sup>
<i>Salix atrocinerea</i>	1.6 $\pm$ 0.1 <sup>b</sup>	0.08 $\pm$ 0.001 <sup>b</sup>	37.7 $\pm$ 2.0 <sup>b</sup>	44.8 $\pm$ 2.9 <sup>c</sup>	7.73 $\pm$ 1.61 <sup>c</sup>

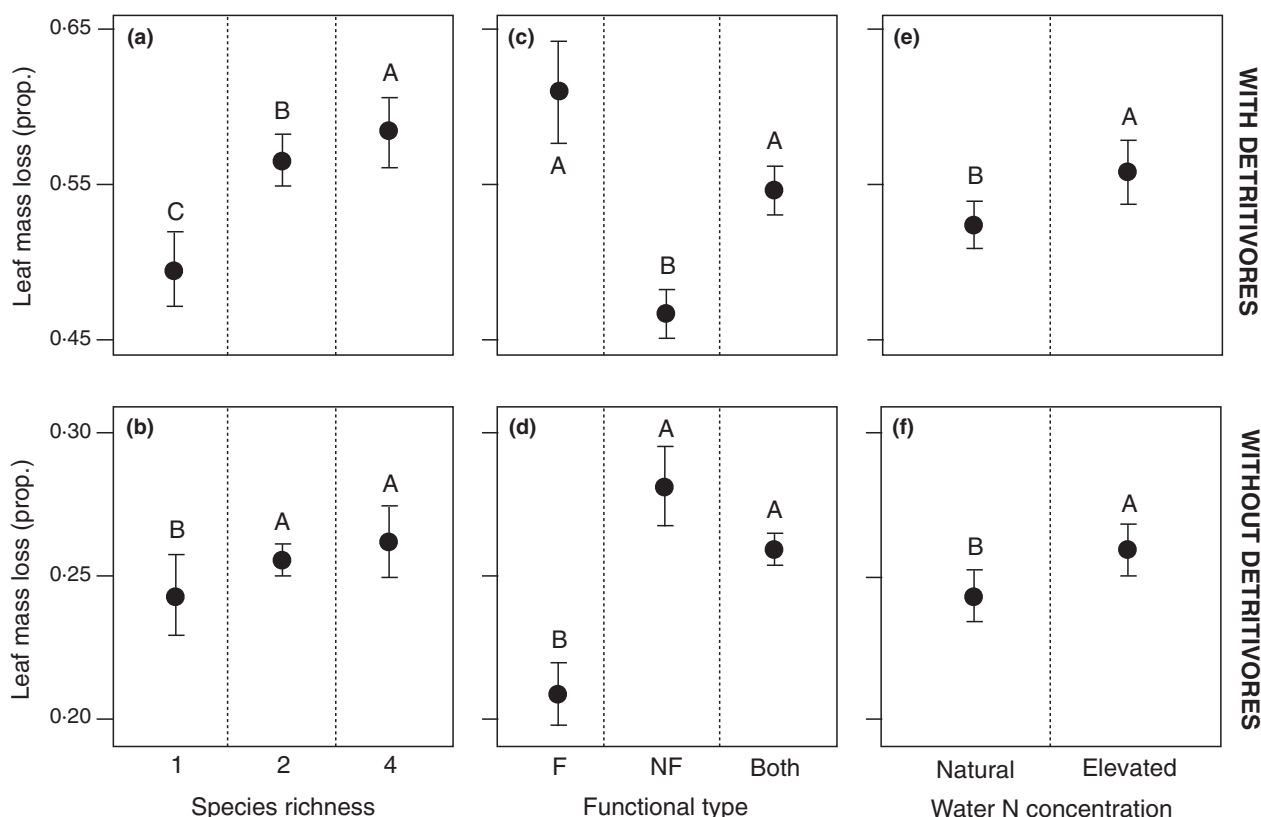
**Table 2.** Results of linear models testing for effects of plant species richness (one, two or four species), functional type (N-fixer, non-fixer or both types), water N concentration (natural or elevated) and interactions on relative litter mass loss (LML) in microcosms with and without detritivores (numDF = numerator degrees of freedom; total degrees of freedom: 110)

Term	numDF	F	P
<b>With detritivores</b>			
Intercept	1	6518.7	<0.0001
Species richness (I)	2	13.6	<0.0001
Functional type (II)	2	65.0	<0.0001
Water N concentration (III)	1	15.4	0.0002
I × III	2	3.0	0.0534
<b>Without detritivores</b>			
Intercept	1	8886.7	<0.0001
Species richness	2	19.6	<0.0001
Functional type	2	47.7	<0.0001
Water N concentration	1	15.9	<0.0001

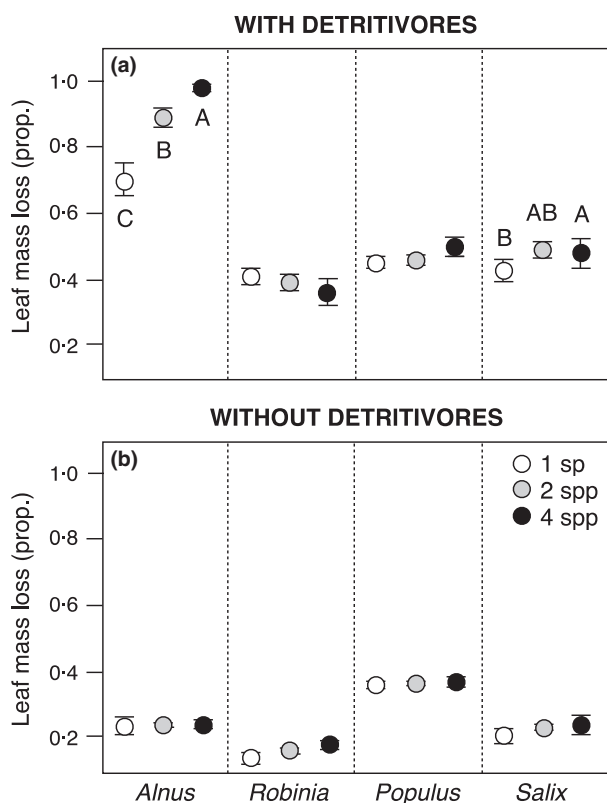
it was higher in microcosms with elevated N concentration (Fig. 2e). As there was a suggestion of weak interaction between species richness and water N concentration ( $P = 0.053$ ; Table 2), we examined the difference between species richness levels separately for natural and elevated N concentrations: at natural N concentration, results were similar to those of total effects; at elevated N concentration, higher LML was only observed for four vs. one species.

When we examined the effect of species identity on LML we found significant differences ( $F_{3,203} = 1701.1$ ,  $P < 0.001$ ): LML was highest for *Alnus* (on average, 84%), intermediate for *Salix* (48%) and *Populus* (47%) and lowest for *Robinia*, (39%) (Fig. 3a). Moreover, there was a significant species identity × species richness interaction ( $F_{6,203} = 6.8$ ,  $P < 0.001$ ) showing that LML increased with species richness only for *Alnus* (two vs. one, four vs. one and four vs. two species) and *Salix* (four vs. one species), and a significant species identity × N concentration interaction ( $F_{3,203} = 6.4$ ,  $P < 0.001$ ), indicating that only *Populus* decomposed faster with elevated N concentration.

When detritivores were absent, LML was also affected by plant species richness, plant functional group and water N concentration (Tables 2, S1): LML increased with two vs. one and four vs. one species (Fig. 2b); was higher for non-fixers and for both functional types together than for N-fixers (Fig. 2d); and was higher at elevated N concentration (Fig. 2f). Species identity also affected LML ( $F_{3,197} = 239.3$ ,  $P < 0.001$ ); LML was highest for *Populus* (on average, 37%), intermediate for *Alnus* (24%) and *Salix* (23%), and lowest for *Robinia* (16%) (Fig. 3b). The leaching trial performed after the experiment showed that LML due to leaching was highest for *Populus* (on average, 29%), intermediate for *Alnus* (21%) and *Robinia* (21%) and lowest for *Salix* (16%) ( $F_{3,16} = 33.4$ ,  $P < 0.001$ ; Table S2).



**Fig. 2.** Relative litter mass loss (LML; mean  $\pm$  SE) in relation to (a, b) species richness (one, two or four species); (c, d) functional type (F = N-fixer, NF = non-fixer or both) and (e, f) water N concentration (natural or elevated), in the presence (a, c, e) or absence (b, d, f) of detritivores. Different capital letters indicate significant differences between treatments.



**Fig. 3.** Relative litter mass loss (LML; mean  $\pm$  SE) of each plant species (*Alnus glutinosa*, *Robinia pseudoacacia*, *Populus nigra* and *Salix atrocinerea*) at different levels of species richness (one, two or four species) in the presence (a) and absence (b) of detritivores. Different capital letters indicate significant differences between treatments.

#### CONTRIBUTION OF COMPLEMENTARITY AND SELECTION TO LITTER MIXING EFFECTS

Net diversity effects averaged  $2.93 (\pm 0.43 \text{ SE})$ , with the additive partitioning showing that complementarity effects ( $2.41 \pm 0.39$ ) were almost five-fold higher than selection effects ( $0.51 \pm 0.08$ ). All effects were higher when detritivores were present than when they were absent ( $P < 0.001$  in all cases); on average, net diversity effects were 11 times higher when detritivores were present ( $5.32 \pm 0.76$  vs.  $0.47 \pm 0.12$ ), complementarity was 10 times higher ( $4.34 \pm 0.69$  vs.  $0.43 \pm 0.12$ ) and selection was 29 times higher ( $0.98 \pm 0.14$  vs.  $0.03 \pm 0.02$ ).

When detritivores were present, increased species richness (from 2 to 4) resulted in higher net diversity (Fig. 4a), complementarity (Fig. 4b) and selection effects (Fig. 4c). Plant functional type also had positive net diversity effects for N-fixers vs. non-fixers and both types together (Fig. 4d); positive complementarity effects for N-fixers vs. both types together (Fig. 4e); and selection effects, dependent on N concentration (Fig. 4f): at natural N concentration, selection was positive and higher for N-fixers than for non-fixers and both types together; at elevated N concentration, selection was higher for N-fixers and for both

types together (both positive) than for non-fixers (negative) (Tables 3, S1).

When detritivores were absent, net diversity effects depended on water N concentration: at natural concentration, the effect increased but became negative in four-species mixtures; at elevated concentration, the effect was positive in all cases and increased from two- to four-species mixtures (Fig. 4g). Complementarity effects showed the same trend as net diversity effects (Fig. 4h), and selection effects increased with species richness but were very close to zero (Fig. 4i). Plant functional type affected net diversity effects, which were positive in all cases, being higher for non-fixers than for N-fixers (effect close to zero) and intermediate when both types were present (Fig. 4j). Complementarity effects showed a similar trend but there were no significant differences among functional types (Fig. 4k). Selection effects again depended on N concentration (Fig. 4l): at natural concentration the effect was higher (but negative) for N-fixers than for both types together (close to zero), and intermediate (positive) for non-fixers; at elevated concentration, the effect was higher (positive) for both types together than for N-fixers (close to zero) and non-fixers (negative) (Tables 3, S1).

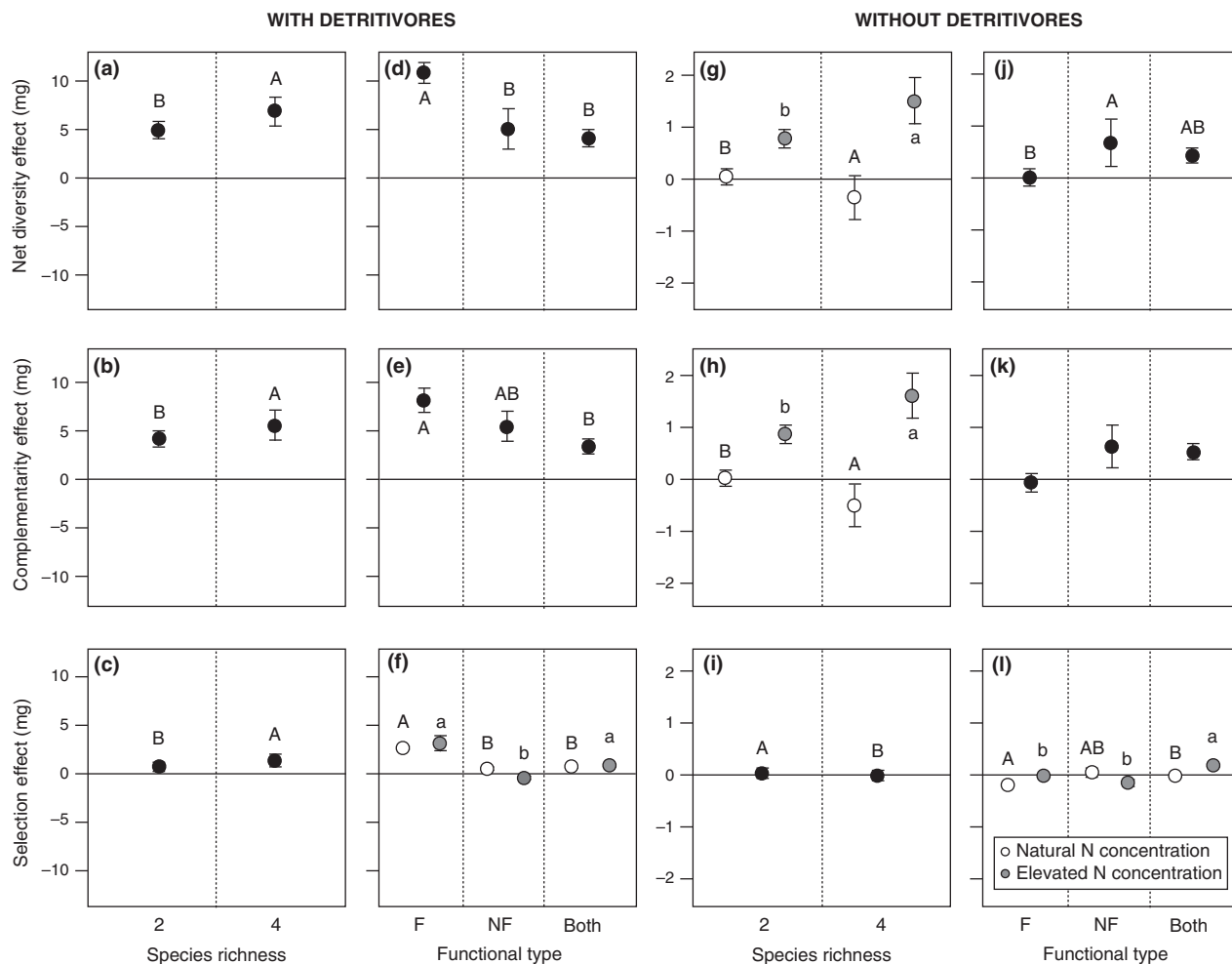
#### DETRITIVORE GROWTH AND C : N RATIOS

Detritivore growth was not affected by plant species richness or functional type, but decreased at elevated N concentration ( $F_{1,110} = 5.3$ ,  $P = 0.0234$ ; Table S1). Detritivore C : N ratios were  $\sim 1.2$  times lower before than after the experiment ( $5.54$  vs.  $6.59$ ;  $t = -2.71$ ,  $P = 0.0078$ ), but they were not affected by plant species richness, functional type or water N concentration (the final model only retained the factor 'water N concentration', which was not significant:  $F_{1,110} = 1.6$ ,  $P = 0.2022$ ; Tables S1 and S3).

## Discussion

#### PLANT LITTER DIVERSITY ENHANCES DECOMPOSITION THROUGH COMPLEMENTARITY EFFECTS

Our results showed that decomposition was faster for litter mixtures than for monocultures, supporting our first hypothesis that litter species richness would promote decomposition. This occurred whether detritivores were present or absent, indicating that microbial decomposers (and possibly detritivores) increased their activity at higher levels of litter diversity. This result contrasts with some previous reports that litter mixing influences detritivores but not microbial decomposers (Swan & Palmer 2004; Sanpera-Calbet, Lecerf & Chauvet 2009). A possible mechanism behind litter mixture effects on microbial decomposition is the active microbial transfer of nutrients among litter types (Gessner *et al.* 2010), including transfer from litter of N-fixing plants to that of non-fixing, rapidly decomposing plants (Handa *et al.* 2014). Although we



**Fig 4.** Net diversity effects (top panels), complementarity effects (middle panels) and selection effects (bottom panels) of plant litter mixtures (mean  $\pm$  SE) on LML for different levels of species richness (a, b, c, g, h, i), functional type (d, e, f, j, k, l) and water N concentration (different coloured dots in panels f, g, h, l). Explanation of treatments as in Fig. 1. Different capital letters indicate significant differences between treatments; when the species richness (or functional type)  $\times$  water N concentration interaction was significant, capital and non-capital letters were used to denote significant differences within each water N concentration.

were unable to explore the mechanisms behind litter mixing effects on microbial decomposition, we showed that these effects could vary depending on nutrient concentration in the water, as explained below.

Nevertheless, detritivores played an important role in mediating diversity effects, which were more than 10 times stronger in the presence of detritivores than in their absence. Moreover, when detritivores were present, diversity effects were always stronger at higher levels of diversity (i.e. in four-species litter mixtures compared to two-species mixtures), and were mostly due to positive complementarity effects. Positive complementarity can occur through resource partitioning or synergistic interactions (facilitation), although it is difficult to distinguish between these mechanisms (Loreau & Hector 2001). Our results demonstrate, however, that increased rates of decomposition in litter mixtures were not linked to enhanced detritivore growth, thus not supporting our hypothesis of a balanced diet effect. It is possible that all litter combinations

provided sufficient resources for maximum detritivore growth in all cases (Boersma & Elser 2006), or the low concentration of phosphorus may have prevented growth (Frost *et al.* 2006).

#### PLANT N-FIXING CAPACITY DOES NOT DRIVE DIFFERENCES IN DECOMPOSITION

Our results only partly supported our second hypothesis, which predicted that litter of N-fixers would decompose faster than that of non-fixers and that detritivore growth would be higher on N-fixers. Detritivore growth was similar between functional types, and decomposition was higher on N-fixers only when detritivores were present, mostly because detritivores preferentially fed on *Alnus*, which had the highest quality leaves (greatest N and P content and lowest ash content). *Alnus* is known to decompose faster than many other riparian species, with and without detritivores (Hladysz *et al.* 2010; Bruder *et al.*

**Table 3.** Results of linear models testing for effects of plant species richness (one, two or four species), functional type (N-fixer, non-fixer or both types), water N concentration (natural or elevated) and interactions on net diversity, complementarity and selection effects in microcosms with and without detritivores (numDF = numerator degrees of freedom; total degrees of freedom of model with detritivores = 71; total degrees of freedom of model without detritivores = 69)

Term	numDF	F	P
With detritivores			
Net diversity			
Intercept	1	75.8	<0.0001
Species richness	1	12.9	<0.0001
Functional type	2	26.7	<0.0001
Complementarity			
Intercept	1	39.2	<0.0001
Species richness	1	6.7	0.0119
Functional type	2	9.9	0.0002
Selection			
Intercept	1	81.4	<0.0001
Species richness (I)	1	29.6	<0.0001
Functional type (II)	2	44.6	<0.0001
Water N concentration (III)	1	10.1	0.0023
II × III	2	11.5	0.0001
Without detritivores			
Net diversity			
Intercept	1	19.0	<0.0001
Species richness (I)	1	0.1	0.7941
Functional type (II)	2	4.3	0.0170
Water N concentration (III)	1	17.9	0.0001
I × III	1	5.0	0.0294
Complementarity			
Intercept	1	18.3	0.0001
Species richness (I)	1	0.1	0.7973
Water N concentration (II)	1	10.6	0.0017
I × II	1	4.6	0.0353
Selection			
Intercept	1	71.9	<0.0001
Species richness (I)	1	19.5	<0.0001
Functional type (II)	2	68.1	<0.0001
Water N concentration (III)	1	48.7	<0.0001
II × III	2	5.0	0.0101

2014), and the presence of *Alnus* causes litter mixtures to decompose faster than expected (Leroy & Marks 2006; Taylor, Mallaley & Cairns 2007; Ferreira, Encalada & Graça 2012). In contrast, when detritivores were absent, decomposition was faster on non-fixers, mainly because *Populus* decomposed faster than the other species. *Populus* had the highest C : N ratio and the lowest N : P ratio, suggesting that micro-organisms used these leaves to select P over N and thus overcome possible stoichiometric imbalances (Gessner *et al.* 2010). We note that the higher decomposition of *Populus* could have been partly due to higher leaching, as indicated by the leaching trial conducted a posteriori. However, *Populus* lost on average 51% more mass than other species in the leaching trial, and 80% more mass than other species in experimental microcosms without detritivores; this difference suggests that microbial decomposition was in fact higher for *Populus* than for the other species.

*Robinia* decomposed more slowly than other species. *Robinia* is a North American N-fixing species that has been introduced to many countries (Contu 2012) and is commonly found in riparian forests in the Iberian peninsula (Castro-Díez *et al.* 2011). It is unlikely that the exotic nature of *Robinia* unduly influenced the results, as microbial decomposers and detritivorous caddisflies are typically able to process leaves of mixed provenance (e.g. Hladyz *et al.* 2009; Boyero *et al.* 2012; Makkonen *et al.* 2012). Moreover, its lignin content is generally lower than that of *Salix* and *Populus* (see above). It is possible, however, that *Robinia* litter had higher content of condensed tannins (Horigome, Kumar & Okamoto 1988) that could suppress microbial assimilation and deter detritivores from feeding (Gessner *et al.* 2010). Moreover, *Robinia* had the lowest P content, and its N content was lower than that of *Alnus* and more similar to that of *Salix*. Although we would have expected *Salix* to have lower N concentration than the N-fixing species, others have reported values similar to ours (Escudero *et al.* 1992).

These results suggest that decomposition varied among species because of differences in a combination of litter traits, rather than to their N-fixing capacity alone. Mixtures of litter of N-fixers and non-fixers did not increase decomposition rates or detritivore growth, contrasting with findings of Handa *et al.* (2014), the basis of our third hypothesis. However, we have shown that the presence of more refractory (or less preferred) species in litter mixtures can enhance the decomposition of faster decomposing species, possibly because of a greater concentration of decomposers or detritivores on their preferred resource, as suggested by Sanpera-Calbet, Lecerf & Chauvet (2009). Decomposition of the preferred resource (here *Alnus* and, to a lesser extent, *Salix*) may have been enhanced in litter mixtures compared to monocultures (and in four-species compared to two-species mixtures) because detritivores processed smaller fragments or even minor leaf nerves of the preferred resource as it became scarce, in preference to the mesophyll of the more recalcitrant leaves. However, the decomposition of less preferred resources (*Robinia* and *Populus*) when detritivores were present was not reduced in litter mixtures compared to monocultures, suggesting that detritivores also exploited these resources. This contrasts with evidence of slower decomposition of recalcitrant species in litter mixtures (Swan & Palmer 2006). It is likely that functional evenness of litter mixtures (i.e. the relative abundance of different litter types) is at least as important as the number of litter types or species in mediating litter diversity effects (Sanpera-Calbet, Lecerf & Chauvet 2009).

#### WATER N CONCENTRATION MODULATES PLANT LITTER EFFECTS ON MICROBIAL DECOMPOSITION

Although our results did not support our fourth hypothesis, they showed that litter diversity effects on microbial decomposition were modulated by water N concentration:

net diversity and complementarity effects were positive only at elevated N concentration and became negative in four-species mixtures at natural N concentration. This suggests that microbial nutrient transfer that causes litter diversity effects (Gessner *et al.* 2010; Handa *et al.* 2014) is enhanced when N is readily available in the water.

Faster decomposition at elevated N concentration demonstrated that micro-organisms were able to use N from the water, although the effect was only evident for *Populus*. The fact that *Populus* litter had the lowest N content and N : P ratio in our study, and its decomposition was enhanced at elevated water N concentration, suggests that micro-organisms were able to use N from the water and P from *Populus* litter (P is more easily leached from litter than N; Gessner 1991) and thus overcome nutrient imbalances and maximize decomposition. In any case, we note that as our experiment lacked microbial inoculation, the only source of fungal spores was the stream water, so micro-organisms were probably underrepresented compared to other laboratory experiments (e.g. Ferreira & Chauvet 2011; Gonçalves *et al.* 2014). This might explain the lack of enhanced decomposition at elevated N concentration for most plant species.

Faster decomposition at elevated N concentration was not accompanied by enhanced detritivore growth, which was actually impaired, possibly because nutrient excess (and thus stoichiometric imbalance) can cause metabolic costs through increased excretion rates, slowing down growth even when nutrient availability is higher (Boersma & Elser 2006). C : N ratios did not differ across treatments, but were higher at the end of the experiment, indicating that detritivores had lower N content than initially. This could occur if detritivores initially had higher quality conditioned leaf material from the stream in their guts than the leaf discs offered during the experiment. However, all C : N ratios fell within the range reported for various detritivores (Hladyz *et al.* 2009).

## Conclusions

Overall, our results provide evidence that litter diversity enhances decomposition through complementarity effects, which are mediated by both microbes and detritivores. Although litter mixing effects on decomposition have been shown previously, our results further suggest that (i) microbes are important in mediating diversity effects on decomposition, although detritivore-mediated effects are stronger; (ii) detritivores enhance the decomposition of their preferred resource in litter mixtures but also process less-preferred resources at rates similar to those in monocultures; (iii) the plant N-fixing capacity does not drive differences in decomposition, which rather depends on a combination of litter traits and (iv) water N concentration modulates plant litter diversity effects on decomposition through microbial activity.

Our findings suggest that plant diversity loss in riparian forests would have different consequences for in-stream

litter decomposition depending on the stream nutrient status as well as the nutritional quality of the remaining litter. It is possible, however, that nutrient enrichment of streams causes the homogenization of nutrient contents of different types of litter, with litter C : N and C : P ratios tending to be generally lower and more similar (Manning *et al.* 2016). How these changes in litter nutritional quality would affect plant diversity effects on microbial and detritivore-mediated decomposition remains unexplored. We have shown that plant diversity effects on decomposition mediated by detritivores are stronger than those mediated by micro-organisms, but microbial processes could become important in streams of high nutrient status, where detritivore populations might be impaired (Woodward *et al.* 2012). Laboratory experiments like ours are indicative of likely scenarios, but are limited by the selection of species and treatments. Comparable in-stream experiments are the next step in understanding real world scenarios and, ideally, would need to be run at multiple sites globally to enable broad generalisations about the results (cf. Boyero *et al.* 2011).

## Authors' contributions

L.B. and J.Po designed the study; A.M.T., S.M., L.B., J.Po and A.B. conducted the experiment; J.Pe and A.M.T. conducted the chemical analyses; A.M.T. and L.B. analysed the data and wrote the manuscript with contribution from all other authors.

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## Data accessibility

Data are available in the electronic supplementary material and the Dryad Digital Repository: <https://doi.org/10.5061/dryad.1k7tr> (Tonin *et al.* 2017).

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## Supporting Information

Details of electronic Supporting Information are provided below.

**Fig. S1.** Leaf mass loss in experimental microcosms with plant monocultures (black circles) and in replicates of the leaching trial conducted several months after the experiment (red circles).

**Fig.S2.** Boxplots of relative leaf mass loss (a), net diversity (b), complementarity (c) and selection effects (d) in relation to detritivore presence.

**Fig. S3.** Relative leaf mass loss in different microcosms.

**Table S1.** Summary of backward model selection based on the Akaike information criterion (AIC) for leaf mass loss, net diversity effects, complementarity effects and selection effects in microcosms with and without detritivores, and detritivore growth and C : N ratios in microcosms with detritivores.

**Table S2.** Mean ( $\pm$ SE) relative leaf mass loss due to leaching for each plant species.

**Table S3.** Mean ( $\pm$ SE) detritivore case length (mm), initial biomass (mg; sum of the three individuals in each microcosm), carbon : nitrogen ratio (C : N) and growth rate (proportion) for each plant species combination, plant species richness level, plant functional type and water N concentration.

**Table S4.** Summary of linear model testing for differences in initial biomass of detritivores subjected to different treatments of plant species richness, plant functional type and water N concentration.

**Table S5.** Mean ( $\pm$ SE) contribution of detritivores to leaf mass loss (prop.) of different plant species combinations.