Hydrobiologia (2018) 806:333–346
https://doi.org/10.1007/s10750-017-3372-3

PRIMARY RESEARCH PAPER

Responses of microbially driven leaf litter decomposition to stream nutrients depend on litter quality

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Received: 3 February 2017 / Revised: 29 August 2017 / Accepted: 31 August 2017 / Published online: 15 September 2017

Abstract The present study aims to understand how microbial decomposition of leaf litter from two riparian tree species differing in their quality varies among streams covering a gradient of nutrient concentrations. We incubated leaf litter from alder (*Alnus glutinosa*) and sycamore (Platanus × hispanica) in 3 streams with low human pressure and 2 streams influenced by wastewater treatment plant effluents. We quantified leaf litter decomposition rates (k) and examined the temporal changes in the leaf litter concentrations of carbon (C) and nitrogen (N) throughout the incubation period. We measured the extracellular enzyme activities involved in degradation of C (i.e., cellobiohydrolase) and organic phosphorus (i.e., phosphatase). Results showed that alder k decreased with increasing nutrient concentrations, while sycamore decomposed similarly among streams. For both species, leaf litter N concentrations were positively related to in-stream dissolved N concentrations. However, we found different temporal patterns of leaf litter N concentrations between species. Finally, we found relevant differences in the enzymatic activities associated to each leaf litter species across the nutrient gradient. These results suggest that the intrinsic characteristics of the leaf litter resources may play a relevant role on the microbially driven leaf litter decomposition and mediate its response to dissolved nutrient concentrations across streams.

Keywords Stream · Leaf litter decomposition · Leaf litter quality · Nitrogen · Phosphorus · Microbial exoenzymatic activity

Electronic supplementary material The online version of this article (doi:10.1007/s10750-017-3372-3) contains supplementary material, which is available to authorized users.

Handling editor: Verónica Jacinta Lopes Ferreira

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Introduction

Decomposition of leaf litter is a fundamental process in streams since it contributes to the metabolism (Webster & Benfield, 1986; Tank & Webster, 1998; Wallace et al., 1999), nutrient cycling (Tank et al., 2000), and food webs (Fisher & Likens, 1973; Vannote et al., 1980) of these ecosystems. Microbial

assemblages (mainly fungi and bacteria) in streams can use leaf litter as a colonizing substrate as well as a source of carbon (C) and nutrients for their development and metabolic activity. In addition, microbial assemblages on leaf litter can also meet their nutrient demand from dissolved compounds in the stream water column (Suberkroop & Chauvet, 1995; Gulis & Suberkroop, 2003). Therefore, both leaf litter quality and nutrient concentrations in streams are expected to influence microbial growth and activity on decomposing leaf litter, which ultimately can dictate their decomposition rates (Webster & Benfield, 1986; Gulis & Superkropp, 2003).

Quality of leaf litter is commonly assessed by its elemental composition (i.e., the concentration of C, nitrogen [N], and phosphorus [P]) and the relative proportions among these elements (Melillo et al., 2001). In general, leaf litter with high N and P concentrations relative to C concentration decomposes faster than leaf litter with low relative concentration of N and P (Webster & Benfield, 1986; Enriquez et al., 1993). Other indicators of leaf litter quality are related to the toughness of the leaves, the presence of wax products, and the complexity of organic C molecules that constitute the leaves (Webster & Benfield, 1986). Simple organic compounds in leaf litter, such as soluble polysaccharides, are labile C sources, and thus are easily degraded and consumed by microbes. In contrast, more complex C compounds in leaf litter, such as lignin or tannins, are recalcitrant C resources, and thus metabolically more costly to be used by microbes (Sinsabaugh et al., 1993). Therefore, relatively higher proportions of recalcitrant C sources in leaf litter have been negatively related to leaf litter decomposition rates (Schindler & Gessner, 2009).

Extracellular enzyme production is the primary mechanism by which fungi and bacteria degrade polymeric and macromolecular compounds from organic matter into low-molecular-weight (LMW) molecules. LMW molecules can then be assimilated by microbial communities (Rogers, 1961). In this sense, microbial activity associated with decomposing leaf litter is commonly assessed by extracellular enzyme activities (Sinsabaugh et al., 1994; Romaní et al., 2006). The most relevant extracellular enzyme activities involved in leaf litter decomposition are those related to the degradation of cellulose (such as β-glucosidase and cellobiohydrolase), hemicellulose (such as β-xylosidase), and lignin (such as phenol oxidases). In addition, N- and P-containing organic compounds are degraded by the activities of peptidases and phosphatases, respectively (Sinsabaugh et al., 1993; Romaní et al., 2006). The activity of these extracellular enzymes can be also influenced by the nutrient availability and the relative proportions between nutrients in the stream, since these enzymes can also degrade compounds from the water column (Sala et al., 2001; Romaní et al., 2004, 2012; Sabater et al., 2005; Romaní et al., 2012).

Inorganic nutrients from the water column can be additional sources of energy and matter to microbial assemblages on leaf litter (Suberkroop & Chauvet, 1995; Hall & Meyer, 1998; Ferreira et al., 2015). Therefore, differences in dissolved nutrient concentrations could explain part of the observed variability in decomposition rates for a given leaf litter type across streams (Webster & Benfield, 1986; Woodward et al., 2012). The stimulation of leaf litter decomposition by nutrient concentrations has been observed in response to increasing concentrations of dissolved inorganic N (DIN) (Richarson et al., 2004), P (Rosemond et al., 2002), and combined enrichment of N and P (Gulis & Superkropp, 2003; Rosemond et al., 2015). In contrast, other studies reported that decomposition rates were not stimulated by nutrient enrichment, especially when background nutrient concentrations (i.e., before the nutrient enrichment) were not limiting (Royer & Minshall, 2001; Chadwick & Huryn, 2003; Albelho & Graça, 2006; Baldy et al., 2007). Furthermore, leaf litter decomposition rates can be lowered in polluted streams, probably because other factors may counteract the stimulating effects of nutrient enrichment on leaf litter decomposition (Webster & Benfield, 1986; Pascoal & Cássio, 2004; Woodward et al., 2012). The relationship between microbially driven leaf litter decomposition rates and nutrient concentrations has been also described by Michaelis–Menten models (Gulis et al., 2006; Pereira et al., 2016) suggesting that other factors beyond the nutrient concentrations may limit leaf litter decomposition rates in streams. Moreover, contrasting results among studies examining the effect of nutrient concentrations on leaf litter decomposition could be also explained by leaf litter quality, which may dictate the strength of interactions between microbial assemblages and

dissolved nutrients. In this sense, a recent meta-analysis showed that the magnitude of the nutrient enrichment effect on leaf litter decomposition was usually higher for leaf litter with low and intermediated N concentrations such as *Quercus* than for high-N litter such as *Alnus* (Ferreira et al., 2015). However, in other cases the decomposition of nutrient-poor *Fagus* or *Eucalyptus* leaf litter was not affected by nutrient enrichment, suggesting that other factors beyond the litter N concentration may influence the effect of nutrient enrichment on leaf litter processing in streams (Ferreira et al., 2015).

The present study aims to understand how microbially driven decomposition of leaf litter from two riparian tree species differing in elemental composition (i.e., C:N ratio) varies among streams which cover a gradient of nutrient concentrations. To approach this question, we incubated leaf litter from alder *Alnus glutinosa* (low C:N ratio) and sycamore Platanus × hispanica (high C:N ratio) in 5 different streams. In each stream, we assessed leaf litter decomposition rates, leaf litter C and N concentrations throughout the decomposition period, and microbial extracellular enzyme activities of cellobiohydrolase (*cbh*) and phosphatase (*phos*) after 85 days of leaf litter incubation. We expected (a) that leaf litter decomposition rates would increase with nutrient concentrations, and (b) to find a larger effect of nutrient concentrations on decomposition for the low-quality leaf litter species (i.e., sycamore) if nutrients in the water column act as an important additional energy and matter sources to microbial assemblages developing on leaf litter.

Methods

Study sites

This study was performed in 5 streams located in different tributaries of La Tordera catchment (Catalonia, NE Spain, Table 1). Three of them are streams with low human influence (Llavina-LLAV, Santa Fe-SF, and Font del Regàs-FR; Table 1), and thus are characterized by relatively low nutrient concentrations (von Schiller et al., 2008). The other 2 streams (Gualba-GUAL and Santa Coloma-COL; Table 1) receive the inputs from wastewater treatment plants (WWTP), and thus, these streams have higher

Table 1 Longitudinal (Long.) and latitudinal (Lat.) location of the streams, average and SEM (in parenthesis, $n = 21$) of physical and chemical variables for each stream during the study period, decomposition rates (k) for alder and sycamore, and the ratio between decomposition rates of both alder and sycamore leaf litter

Stream	Long. 2°E	Lat. 41°N	Discharge ($l\ s^{-1}$)	Temp. (°C)	NO_3 ($\mu g\ N\ l^{-1}$)	NH_4 ($\mu g\ N\ l^{-1}$)	SRP ($\mu g\ P\ l^{-1}$)	DIN ($\mu g\ N\ l^{-1}$)	k alder (dd^{-1})	k sycamore (dd^{-1})	k alder: k sycamore ratio
SF	27′52″	46′37″	67 (29)^{a}	5.3 (0.1)^{a}	39 (13)^{a}	13 (3)^{a}	13 (2)^{a}	51 (13)^{a}	0.00132^{A-a}	0.00085^{A-a}	1.55
FR	27′00″	49′32″	67 (14)^{a}	6.5 (0.2)^{b}	150 (26)^{ab}	19 (4)^{a}	5 (1)^{a}	169 (27)^{ab}	0.00131^{A-a}	0.00066^{A-b}	1.98
LLAV	23′52″	45′09″	224 (113)^{b}	6.7 (0.1)^{ab}	261 (47)^{ab}	27 (8)^{a}	9 (1)^{a}	288 (45)^{b}	0.00148^{A-a}	0.00067^{A-b}	2.21
GUAL*	30′17″	44′02″	155 (33)^{b}	7.3 (0.2)^{ab}	307 (40)^{b}	471 (8)^{b}	75 (12)^{ab}	778 (100)^{c}	0.00093^{A-a}	0.00058^{A-a}	1.60
COL*	39′32″	51′48″	156 (37)^{b}	9.4 (0.2)^{b}	1549 (127)^{c}	941 (288)^{c}	103 (47)^{b}	2490 (224)^{d}	0.00064^{B-a}	0.00053^{A-a}	1.21

Different letters indicate significant differences on k based on one-way ANCOVA analysis and in the rest variables based on ANOVA analysis, followed by post hoc Tukey's t test. Note that for k capital and lower case letters indicate statistical differences among streams and between leaf litter species, respectively. DIN = dissolved inorganic nitrogen (nitrite + nitrate + ammonia). Streams influenced by wastewater treatment plant inputs are indicated with asterisks

nutrient concentrations. In these streams, nutrient enrichment could potentially enhance leaf litter decomposition rates. However, in many cases WWTP effluents also contain other pollutants, such as barium or aluminum, that may have the opposite effect on leaf litter decomposition (Pascoal & Cássio, 2004; Woodward et al., 2012). All the study sites are second- and third-order streams, with relatively well-preserved stream channel morphology characterized by riffles and pools. All the streams are flanked by riparian forest dominated by alder (*Alnus glutinosa* (L.) Gaertn.), black poplar (*Populus nigra* L.), and sycamore (Platanus x hispanica (Mill.) Münchh), except the SF stream where European beech (*Fagus sylvatica* L.) dominates the catchment as well as the stream banks.

Field experiments

For this study, we used leaves of alder and sycamore as species with high and low quality in terms of C:N ratio, respectively. Leaves from alder and sycamore were collected in November 2010 at GUAL site. To measure litter decomposition rates (k, degree-days^{-1}), we followed procedures by Webster & Benfield (1986). For each leaf litter species, 5 g of air dried leaves was placed in 250-µm-mesh-size bags, which mostly excluded macroinvertebrates and thus basically allowed measurement of microbial leaf litter decomposition. Leaf bags were deployed in the selected streams, anchored on the streambed with metal bars, and incubated in the streams from November 11, 2010 to March 10, 2011. At each stream, three leaf bags for each leaf litter species were collected on days 8, 15, 29, 47, 85, and 119 after deployment. Collected leaf bags were kept cold (~4° C) in the field and in the laboratory until later measurements of dry weight and C and N leaf litter concentrations. On each sampling date, stream water samples were collected to analyze the concentrations of ammonium (N–NH_4^+), nitrite (N–NO_2^-), nitrate (N–NO_3^-), and soluble reactive phosphorus (SRP). We also measured stream discharge based on cross-section measurements of width, water depth, and water velocity (Gordon et al., 2004). At each stream, we continuously recorded water temperature every 20 min during the entire incubation period using temperature data-loggers (HOBO Pendant® UA-002-64) placed on the streambed. After 85 days of leaf litter incubation in the streams, we collected additional leaf bags to quantify the extracellular enzyme activities of cellobiohydrolase (*cbh*; EC 3.2.1.91) and phosphatase (*phos*; EC 3.1.3.1-2) as outlined in Romaní et al. (2006). We measured *cbh* activity as an indicator of leaf litter microbial degradation activity and especially for a recalcitrant compound such as cellulose. We measured *phos* activity to assess how changes in the inorganic nutrient availability (i.e., SRP) may affect the potential microbial use of organic phosphorus compounds. We quantified the enzyme activity after 85 days of incubation when the leaf litter packs roughly loosed 40–60% of initial mass. At this point, we expected that microbial assemblages were well developed and extracellular enzyme activities were high (Romaní et al., 2006).

Laboratory methods and data analysis

Stream water samples were analyzed at the Nutrient Analysis Service of the Centre d´Estudis Avançats de Blanes (CEAB) for nutrient concentrations using an Automatic Continuous Flow Futura-Alliance Analyzer and following standard colorimetric methods (APHA, 1998).

In the laboratory, leaf litter samples collected on each sampling date and at each stream were carefully rinsed with stream water to remove inorganic sediment attached to the leaf surface. Then, leaf litter samples were oven-dried until constant weight (60°C for 48 h) and weighed to obtain the remaining dry mass. Sub-samples of leaf litter were ignited (500°C, 4 h) to calculate ash-free dry mass (AFDM), which was expressed as percentage of the initial AFDM. The remaining AFDM on each sampling date for each leaf litter types and for each stream was plotted against degree-days (i.e., summing the daily mean temperature registered along the study period). The relationship fitted a negative exponential model described by Petersen & Cummins (1974):

$$W_t = W_0 * e^{-kdd}, \quad (1)$$

where W_0 and W_t are AFDM (g) at the beginning and at sampling dates, respectively, dd (degree-days) is the incubation time expressed in terms of summed mean daily water temperature (°C) up to the sampling dates, and k is the decomposition rate (expressed in terms of dd^{-1}). Values of k denote the velocity at

which mass of leaf litter decreases over time corrected for the potential temperature differences among streams, so that k values can be compared among sites with different water temperatures.

Concentration of C (g C/g DM) and N (g N/g DM) in leaf litter before and over the incubation period for the 2 leaf litter species and among the 5 study streams were measured for the collected samples. Dried subsamples were ground to a fine powder, and a subsample of 1.5 mg was weighed and encapsulated in tin vials. Samples were sent to the Unidade de Técnicas Instrumentais de Análise (Universidade da Coruña, Spain) for the analysis of elemental C and N concentrations, which was done by sample combustion using an elemental autoanalyzer EA1108 (Carlo Erba Instruments). Data of N concentrations at d 85 were used to explore how the effect of dissolved nutrient concentrations influences on leaf litter N concentrations.

Extracellular enzyme activities of *cbh* and *phos* on leaf litter samples incubated for 85 days were measured using methylumbelliferyl (MUF) fluorescent-linked substrates, following the method described in Romaní et al. (2006). These assays were conducted at saturation substrate conditions of 0.3 mM. Leaf litter disks (14 mm diameter, 3 replicates per experimental condition) and water controls were incubated for 1 h in the dark in a shaker. Blanks and standards of MUF (0–100 µmol L^{-1}) were also incubated. At the end of the incubation, Glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 365/455 nm excitation/emission (Kontron SFM25 fluorimeter). Results of extracellular enzyme activities were expressed as the amount of MUF substrate produced per incubation time (h) and leaf litter ash-free dry mass (AFDM; g).

Statistical analysis

To determine differences in the physical and chemical variables among study streams, we used a one-way analysis of variance (ANOVA) model with stream ($n = 5$) as fixed factor followed by post hoc Tukey's t test. We also used a one-way ANOVA model to determine initial differences in the leaf litter C and N concentrations and the C:N ratio among the 2 leaf litter species.

We used a two-way ANCOVA to explore differences in leaf litter k between the 2 leaf litter species and among the 5 study streams. Fraction of litter remaining AFDM of alder and sycamore was natural log transformed prior to the analysis. The two-way ANCOVA included fraction remaining AFDM as dependent variable, time (expressed in degree-days) as the covariate, and stream ($n = 5$) and leaf litter species ($n = 2$) as fixed factors. We used the interaction term stream*species*degree-days to explore the null hypothesis in which the variability in k among streams did not differ among leaf litter species (Zar, 1999). Additionally, to explore the specific variability of k for each leaf litter species among streams, we also used a one-way ANCOVA for each leaf litter species, which included fraction remaining AFDM as dependent variable, time (expressed in degree-days) as the covariate, and stream ($n = 5$) as a fixed factor. Tukey's test followed significant differences among streams.

To examine differences in the variation in the leaf litter C and N concentrations during the leaf litter decomposition between leaf litter species and across streams, we used two-way ANOVA with repeated measures (RM, i.e., sampling time) with both leaf litter C and N concentrations as dependent variables, respectively, leaf litter species ($n = 2$) and streams ($n = 5$) as fixed factors, and time (expressed in days) as the covariate. In addition, we used linear and asymptotic-type models to explore the best fit of the temporal variation in the N concentrations throughout decomposition period of leaf litter for both alder and sycamore (from November 11, 2010 to March 10, 2011).

The asymptotic model followed the equation:

$$N = \frac{N_{max} d}{K_d + d}, \quad (2)$$

where N_{max} is the maximum leaf litter N concentrations, K_d is the incubation day at which N reach the half of N_{max} concentrations, and d is the incubation time (in days).

We examined differences in extracellular enzyme activities of both *cbh* and *phos* using a two-way ANOVA model with stream ($n = 5$) and leaf litter species ($n = 2$) as fixed factors. We used Pearson correlation coefficients (PCC) to explore relationships between *cbh* and *phos* activities on each leaf litter species. In addition, we explored the

relationships between both *cbh* and *phos* extracellular enzyme activities and the percentage of leaf litter mass loss among streams using data from the d 85 of leaf litter incubation. To do that, we used linear, exponential and asymptotic relationships in order to find the best-fit model.

Finally, to assess differences between leaf litter species in terms of *k*, leaf litter N concentrations, and *cbh* and *phos* activities across increasing nutrient gradient, we explore linear relationships between these parameters and the concentrations of DIN and SRP and the DIN:SRP molar ratio of the study streams for the 2 leaf litter species separately.

Statistical analyses were done with PASW Statistics 18 (v18.0.0/SPSS Inc) and R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). Statistical results were evaluated at the $\alpha = 0.05$ significance level.

Results

Stream characteristics

Stream discharge varied among streams, and was lower in SF and FR than LLAV and the two streams influenced by WWTP effluents (GUAL and COL) (Table 1). Mean water temperature varied 4°C among streams, and was higher in GUAL and COL streams and lowest in SF, the stream located at the highest elevation (Table 1). DIN and SRP concentrations covered a wide range among streams, especially for the DIN species, which spanned two orders of magnitude (Table 1). Concentrations of DIN and SRP were strongly correlated among streams (PCC, $r = 0.90$, P value < 0.001) and both were higher in the streams influenced by WWTP inputs (Table 1). The concentration of NO_3 accounted for the largest fraction of the DIN concentration in all the streams; however, the percentage of DIN as NH_4^+ was higher in the streams influenced by WWTP inputs (Table 1).

Initial leaf litter C and N concentrations and leaf litter decomposition rates

Alder and sycamore leaf litter presented similar C concentrations (44.65 ± 0.56 and 44.60 ± 0.45% of dry mass, respectively) (one-way ANOVA, P value > 0.05). However, alder showed higher N concentrations than sycamore (2.03 ± 0.09 and 1.32 ± 0.12% of dry mass, respectively) (one-way ANOVA, P value < 0.001). Therefore, the C:N ratio of alder leaf litter was significantly lower than the C:N ratio of sycamore leaf litter (one-way ANOVA, P value < 0.001).

On average, *k* values of alder leaf litter were higher than *k* values of sycamore leaf litter (two-way ANCOVA, Tukey's *t* test, P value < 0.001, Table 1). The variability in *k* values among streams was higher for alder than for sycamore leaf litter (Table 1). Among streams, *k* values for both alder and sycamore leaf litter were lower in streams influenced by inputs from WWTP effluents (two-way ANCOVA, Tukey's *t* test, P value < 0.001, Table 1). In addition, in COL (i.e., the stream with the highest nutrient concentrations) we found a smaller difference in *k* between the two leaf litter species (*k* alder: *k* sycamore = 1.21; Table 1). Overall, *k* rate for alder leaf litter was negatively related to stream DIN concentrations ($r^2 = 0.77$, P value < 0.001, Fig. 1A; Table S1) and SRP concentration ($r^2 = 0.93$, P value < 0.001, Table S1). In contrast, no relationships were found between *k* values for sycamore leaf litter and DIN and SRP concentrations (P value > 0.05, Fig. 1B; Table S1). Leaf litter *k* was not related with DIN:SRP molar ratio among streams for neither leaf litter species (Table S1).

Variation in leaf litter C and N concentrations during the decomposition period

The C concentrations did not significantly vary during decomposition period, and values were similar among leaf litter species and among streams (ANOVA-RM, P value > 0.05). In contrast, the N concentrations differed among leaf litter species (ANOVA-RM, P value < 0.01), with alder leaf litter showing higher N concentrations than sycamore leaf litter. The N concentrations of leaf litter during the decomposition period varied among streams (ANOVA-RM, P value < 0.01), with the highest values in COL and lowest values in LLAV. The interaction term (i.e., leaf litter species*stream) of the ANOVA-RM was not significant (P value > 0.05) indicating that differences in N concentrations between alder and sycamore leaf litter during the decomposition period were consistent among streams. The leaf litter N concentrations at d 85 of

incubation period was positively related to stream DIN concentrations for both alder and sycamore leaf litter ($r^2 = 0.66$, P value < 0.01, $r^2 = 0.77$, P value < 0.05, respectively, Fig. 1C, D; Table S1).

The temporal patterns of N concentrations during the decomposition period differed between alder and sycamore leaf litter. The temporal variation of N concentrations in alder leaf litter was best fitted with an asymptotic-type model in all streams (Fig. 2, left panels), except in LLAV (Fig. 2E). N concentrations showed a rapid increase during the early stages of the leaf litter decomposition but then reached a steady state until the end of the incubation period. In contrast, the temporal variation of N concentrations in sycamore leaf litter during the incubation period followed a linear model in all streams (Fig. 2, right panels), except in GUAL (Fig. 2H).

Extracellular enzyme activities

The extracellular enzyme activity of *cbh* was higher for alder than for sycamore leaf litter (2.97 ± 1.6 and 0.57 ± 0.29 µmol MUF g DM^{-1} h^{-1}, respectively; ANOVA, P value < 0.001; Fig. 3A). Values of *cbh* for both alder and sycamore leaf litter significantly differed among streams (ANOVA, P value < 0.001; Fig. 3A). Basically, the higher *cbh* activities for the two leaf litter species were measured in streams with intermediate nutrient concentrations (i.e., LLAV and GUAL). The interaction term of the ANOVA (leaf litter species*stream) was not significant (P value > 0.05), indicating that the variation in *cbh* among streams was consistent among leaf litter species.

Extracellular enzyme activity of *phos* was higher for alder leaf litter than for sycamore leaf litter (8.73 ± 4.33 and 2.30 ± 1.24 µmol MUF g DM^{-1} h^{-1}, respectively; ANOVA, P value < 0.001; Fig. 3B). Values of *phos* for both alder and sycamore leaf litter significantly differed among streams (ANOVA, P value < 0.001; Fig. 3B), and the interaction term (leaf litter species*stream) was not significant (ANOVA, P value > 0.05). Extracellular enzyme activities of *cbh* and *phos* were strongly correlated for both alder leaf litter (PCC, $r = 0.97$, P value < 0.01) and sycamore leaf litter (PCC, $r = 0.95$, P value < 0.01).

Considering data from all streams together, leaf mass loss by d 85 was significantly related to both *cbh* and *phos* activity for alder leaf litter (Fig. 4A, C), but it was not related to any extracellular activity for sycamore leaf litter (Fig. 4B, D). Specifically, for the case of alder leaf litter, we found that the relationship between alder leaf mass loss and enzyme activities of both *cbh* and *phos* was best fitted with an asymptotic-type model ($r^2 = 0.57$, P value < 0.001 and $r^2 = 0.78$, P value < 0.001, respectively, Fig. 4A, C).

Activities of both *cbh* and *phos* did not correlate with concentrations of DIN, SRP, nor the DIN:SRP molar ratio among streams (P value > 0.05, Fig. 1E–H; Table S1). Nevertheless data showed a hump-shape trend characterized by an initial increase of enzyme activities up to 1 mg l^{-1} of DIN followed by a clear decrease above this threshold (Fig. 1E–H).

Discussion

The influence of nutrient gradient on leaf litter decomposition rates

We found that the response of microbially driven leaf litter decomposition rates to the stream nutrient gradient differed between the two leaf litter species considered. This agrees with previous finding (Ferreira et al., 2015) and reinforces the notion that leaf litter quality mediates the responses of leaf litter decomposition to dissolved nutrient concentration in streams. Nevertheless, results do not agreed with our expectations since decomposition rates of alder decreased along the nutrient gradient, while no significant changes were observed in decomposition rates of sycamore across the nutrient gradient. These results suggested that decomposition of high-quality leaf litter (i.e., low C:N ratio), such as alder, may be more sensitive to differences in nutrient concentrations among streams than low-quality leaf litter, such as sycamore. In this sense, Woodward et al. (2012) also found higher variability on decomposition rates for high-quality leaf litter species such as alder than for low-quality litter such as oak across streams covering a 1000-fold nutrient gradient. However, in contrast to our results, their observed responses to increased nutrient concentrations exhibited a hump-shape pattern. Nevertheless, it is worth noting that in Woodward et al. (2012) the significant hump-shape pattern was only observed on total decomposition which includes macroinvertebrate leaf litter breakdown. Other studies focusing on microbial

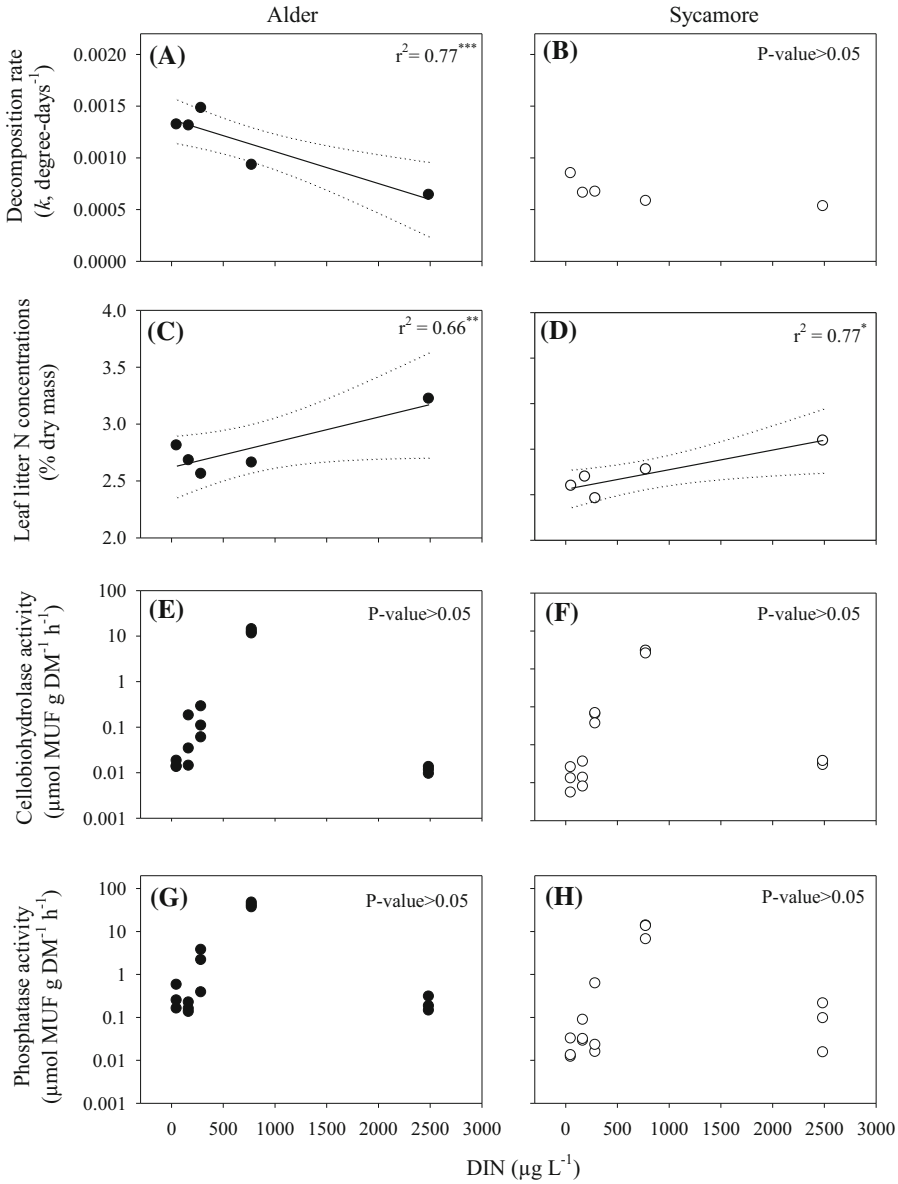

Fig. 1 Relationships between in-stream DIN concentrations and leaf litter decomposition rates (**A**–**B**), the leaf litter N concentrations measured at exposure time of 85 days (**C**–**D**), and the extracellular enzyme activities of both cellobiohydrolase and phosphatase measured at exposure time of 85 days (**E**–**H**). Filled circles (left panels) and open circles (right panels) correspond to data of alder and sycamore leaf litter. Level of significance based on one-way ANOVA analysis is indicated by ***P value < 0.001, **P value < 0.01, and *P value < 0.05. *DIN* dissolved inorganic nitrogen (nitrite + nitrate + ammonia)

decomposition also observed a lack of response of k across stream nutrient gradient (Chauvet et al., 2016). Overall, these results suggest that other factors beyond nutrient concentrations may influence microbial-driven decomposition rates across streams. In this sense, in a recent study conducted under laboratory conditions, Fernandes et al. (2014) found that Michaelis–Menten kinetics best explained the relationship between microbial-driven leaf litter decomposition rates and N availability, suggesting that the activity of microbial assemblages colonizing leaf litter become limited by other factors when N

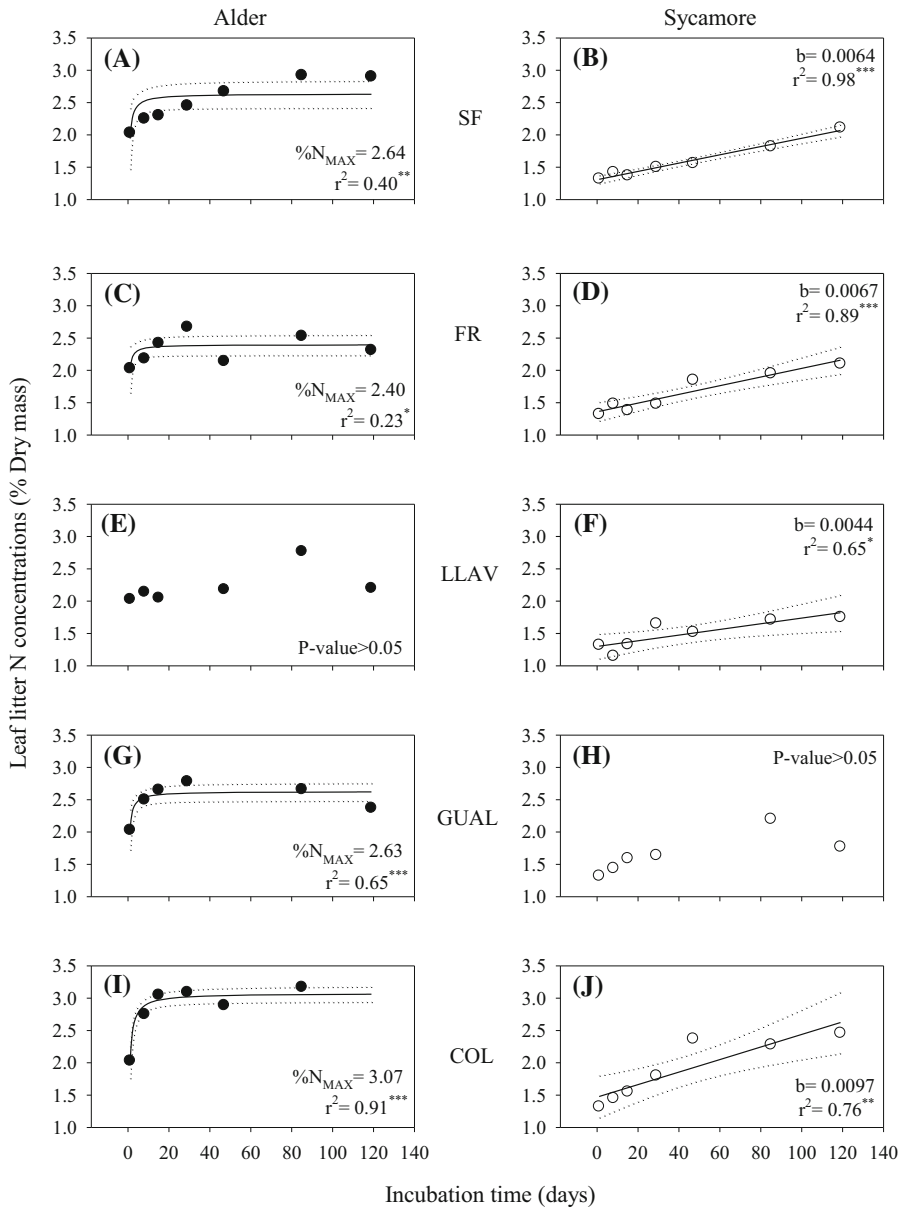

Fig. 2 Temporal variation in the leaf litter N concentrations (as percentage of dry mass) for alder (left panels; asymptotic-type models) and sycamore (right panels; linear models) during the decomposition period in the 5 studied streams. Filled circles (left panels) and open circles (right panels) correspond to data of alder and sycamore leaf litter. N_{max} is the maximum N concentrations on leaf litter during decomposition period (left) and b is the slope of the linear model (right). Level of significance of the models is indicated by ***P value < 0.001, **P value < 0.01, and *P value < 0.05

availability in streams increases as outlined in Bernot & Doods (2005).

We found that microbially driven decomposition of alder was lower in highly polluted streams although it has been reported that nutrient enrichment had a positive or saturating effects on microbial biomass and activity associated with decomposing leaf litter (Suberkropp & Chauvet, 1995; Fernandes et al., 2014), as well as on leaf litter decomposition rates (Fernandes et al., 2014; Ferreira et al., 2015; Rosemond et al., 2015). Our results agree with previous studies showing that on highly polluted streams decomposition is generally reduced regardless of the high stream nutrient concentrations (Pascoal & Cássio, 2004; Lecerf et al., 2006; Woodward et al., 2012). A plausible explanation of these results is that in polluted streams, such as those receiving the effluents from WWTPs, confounding factors may influence the positive effect of nutrient concentrations on leaf litter decomposition (Pascoal & Cássio, 2004; Woodward et al., 2012). In fact, in our WWTP-influenced streams the relatively

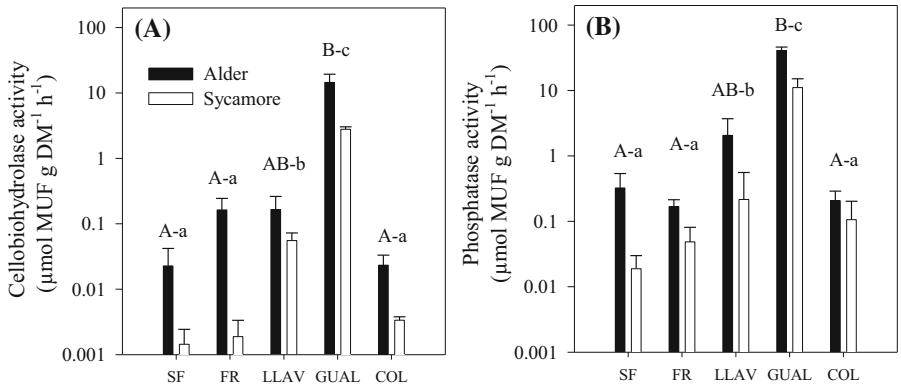

Fig. 3 Extracellular enzyme activities of cellobiohydrolase (left) and phosphatase (right) (+SEM, $n = 3$ per experimental condition) measured on alder and sycamore leaf litter at incubation time of 85 days. Significant differences among streams for alder and sycamore leaf litter species are shown as different capital and lower case letters, respectively, based on two-way ANOVA analysis. Note that streams are ordered following the increasing gradient of DIN concentration, with SF being the stream with the lowest concentration and COL the stream with the highest concentration

proportion of NH_4^+ with respect to total DIN concentrations was higher with respect to that in more pristine streams. A previous study found that NH_4^+ may inhibit leaf litter decomposition rates (Lecerf et al., 2006). Furthermore, WWTP effluents are sources of other compounds such as metals and emergent pollutants, which may have negative effects on the microbial communities, as well as on leaf litter decomposition rates (Webster & Benfield, 1986; Pascoal & Cássio, 2004; Ferreira et al., 2016). Thus, in WWTP-influenced streams these factors could potentially counterbalance the positive effects of nutrient enrichment on leaf litter decomposition leading to the decrease of organic matter decomposition (Kaushik & Hynes, 1971; Pascoal & Cássio, 2004; Woodward et al., 2012).

Differences between leaf litter species during the decomposition period

Decomposition rates of alder leaf litter were consistently higher than those of sycamore leaf litter, regardless of the stream, suggesting that the intrinsic characteristics of the leaf litter may also drive to some extend *k*. This pattern may be related to the higher N concentration, as well as low concentration of refractory compounds such as lignin on alder leaves with respect to that of sycamore (Webster & Benfield, 1986; Gessner & Chauvet, 1994; Cornwell et al., 2008). Nevertheless, in this study, the differences in decomposition rates between alder and sycamore leaf litter were smaller than in other studies (Webster & Benfield, 1986), which could be in part attributed to the lower C:N ratio of sycamore leaf litter (34 ± 0.5) comparing to values reported previously (C:N = 73.6; Gessner & Chauvet, 1994). Nevertheless, we found that the difference in decomposition rates between the two leaf litter species decreased among streams as nutrient concentrations and pollution conditions increased. This suggests that in polluted streams, environmental conditions seem to be more relevant than specific characteristics of the leaf litter on determining the rates of organic matter decomposition.

Alder and sycamore N concentrations at later stages of decomposition period increased as DIN concentrations in streams increased, suggesting that the availability of DIN in streams can influence the activity of microbial assemblages on leaf litter (Molinero et al., 1996; Pozo et al., 1998; Tank et al., 2000; Gulis & Suberkropp, 2003). This response contrasted with that observed for leaf litter decomposition, pointing that mechanisms controlling N concentrations of the microbial leaf litter complex during the decomposition could be independent of the efficiency at which leaf litter mass is lost. However, differences between leaf litter species were highlighted by the different models describing the temporal variation of leaf litter N concentrations between species. These results suggest that, regardless of the stream conditions, leaf litter quality is a relevant factor controlling the dynamics of microbial

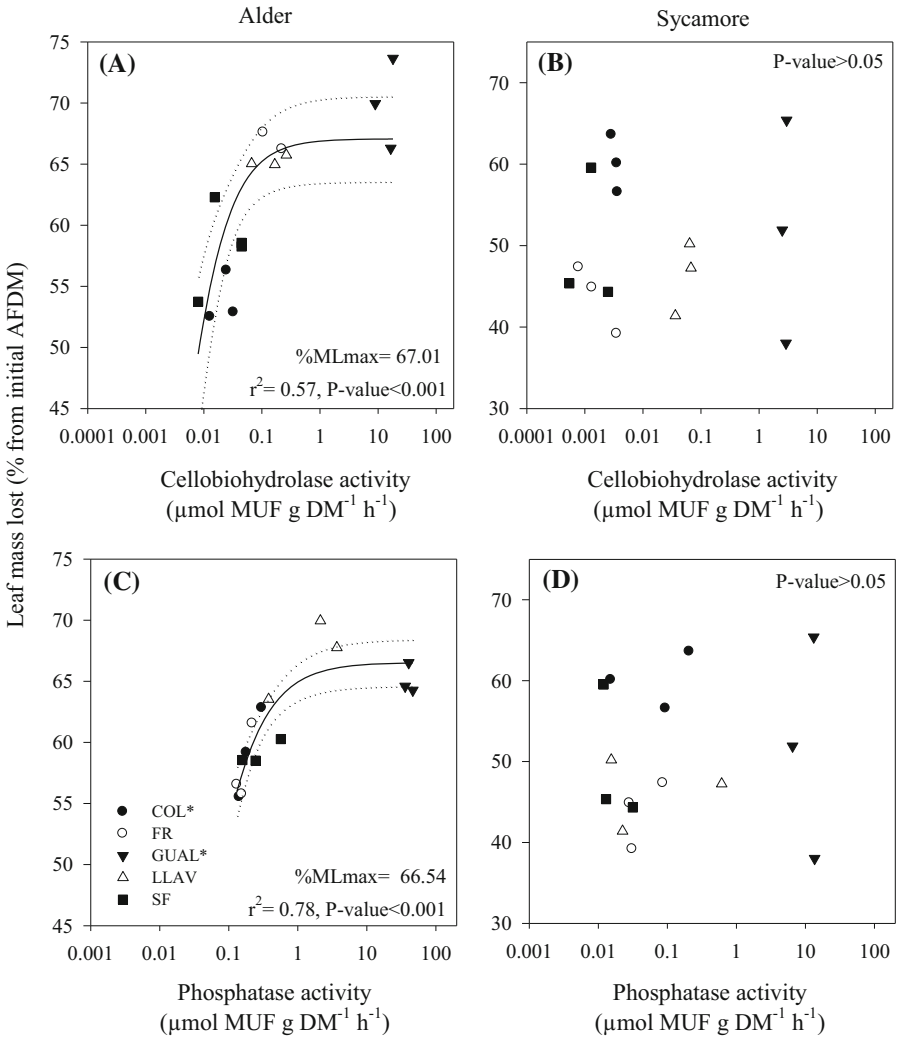

Fig. 4 Relationships between the percentage of leaf litter mass lost on 85 days of leaf litter incubation and the microbial activities of both cellobiohydrolase (up panels) and phosphatase (down panels) for the two leaf litter species, considering data from all streams together. Data from alder leaf litter (left panels) was best fitted by an asymptotic-type model, where %MLmax is the maximum alder mass lost among streams from the model

colonization on leaf litter. Microbial colonization may be faster in high-quality leaves, such as alder, than in low-quality leaves, such as sycamore. These results are in agreement with previous studies about microbial colonization patterns of leaf litter differing in nutrient concentration (Webster et al., 2009) or in the content of recalcitrant compounds (Gessner & Chauvet, 1994), which are factors that can limit growth of fungi on leaf litter (Canhoto & Graça, 1999).

The influence of nutrient gradient on enzyme activities

The variability of *cbh* and *phos* enzyme activities was remarkable among streams and observed patterns were consistently similar for the two leaf litter species, suggesting that water column characteristics can influence the enzymatic activity of microbial assemblages coating leaf litter. We found that *cbh* and *phos* increased as DIN concentration increased;

however, at DIN concentration $>$ 1mgN L^{-1} the two enzymatic activities were significantly depressed. *Cbh* and *phos* are catabolic enzymes, and their expression can be regulated by organic compounds from the leaf litter as well as by chemical compounds from stream water column (Sala et al., 2001; Romaní et al., 2004, 2012). In fact, Sinsabaugh et al. (2005) found that increases in DIN availability lowered *cbh* activity in leaf litter, which is to some extent, in agreement with our results. A similar trend was also found for stream water SRP availability and *phos* activity (Romaní et al., 2004, 2012; Allison & Vitousek, 2005). Overall, these results suggest that enzymatic responses depend on the nutrient availability. In addition, other compounds such as pollutants coming from the WWTPs inputs could also affect extracellular enzyme activities of microbial assemblages (Webster & Benfield, 1986; Freeman & Lock, 1992). In COL, the presence of these compounds could have lowered the *cbh* and *phos* activities and by extension the decomposition rates (Pascoal & Cássio, 2004; Woodward et al., 2012).

The activity associated to cellulose and organic phosphorus decomposition was consistently lower in microbial assemblages growing on sycamore leaf litter than in those growing on alder leaf litter. This pattern also supports the clear effect of leaf litter quality on the activity of the microbial assemblages decomposing organic matter. This agrees with previous studies showing lower values of *cbh* activity in sycamore leaf litter in comparison to alder leaf litter (Artigas et al., 2004) or other nutrient-rich leaf litter species such as black poplar (Artigas et al., 2011). Other studies have attributed the lower values of enzyme activities in sycamore to the higher lignin and tannin concentration of these leaves (Gessner & Chauvet, 1994).

We found that enzyme activities were related with leaf litter mass loss only for alder. This result suggests that leaf litter quality could regulate the enzyme efficiency involved in the leaf litter mass loss across streams. Nevertheless, the highest values of both activities observed in GUAL stream were not related to higher mass loss on alder. In this stream, microbial enzymatic activity could be fueled by a combination of leaf litter resources and water column nutrients, which may explain why the increase of microbial activity did not result in a stimulation of leaf litter mass loss (Suberkroop & Chauvet, 1995). In contrast, the weak relationship between enzyme activities and mass loss in sycamore leaf litter suggested that other enzymes, such as phenol oxidases, may be a limiting step for the decomposition of the leaf tissues. Overall, these findings suggest that enzymatic activity of *cbh* and *phos* of microbial assemblages developing on sycamore leaf litter could be also fueled by dissolved organic sources from water column. Additionally, results suggest that the decomposition of sycamore leaf litter is more limited by the quality of this leaf litter than by the availability of external resources.

Conclusions

Alder and sycamore leaf litter consistently showed different decomposition rates, temporal dynamics of leaf litter N concentrations, and enzyme efficiency of microbial decomposers across the stream nutrient gradient. These results suggest that the influence of stream environmental characteristics on particulate organic matter decomposition may depend on the quality of leaf litter where microbial assemblages develop. Nevertheless, our study suggests that stream characteristics can also negatively influence organic matter decomposition, especially in those streams affected by pollution from WWTP effluents. Overall, the present study suggests that the riparian species composition may play a relevant role on leaf litter decomposition in streams. However, this role could be less clear in polluted streams such us those receiving inputs from WWTPs where leaf litter decomposition and associated microbial activity seem to be inhibited. In conclusion, vegetation with high-quality leaf litter (i.e., alders)-dominating riparian forest could provide a more bioavailable leaf litter substrate for in-stream microbes. In contrast, vegetation with low-quality leaf litter (i.e., sycamore) may provide a less bioavailable decomposing substrate for microbial assemblages, which could grow and develop their enzymatic activity uncoupled to leaf litter mass loss and, thus, to the dynamics of organic matter decomposition across streams.

Acknowledgements The authors would like to thank R. Ventosa for the chemical analyses done at the Nutrient Analysis Service of CEAB. We thank the anonymous

reviewers whose comments contributed to improve the present study. The financial support was provided by the Spanish Ministry of Science and Innovation through the MED-FORESTREAM (ref: CGL2011-30590-C02-02) project. E. Bastias was granted by a FPI PhD fellowship from the Spanish Ministry of Science and Innovation through the MED-FORESTREAM project. M. Ribot was supported by a technical training contract also associated to MED-FORESTREAM project. Funding was provided by Consejo Superior de Investigaciones Científicas (Grant No. BES-2012-053358).

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