AIL

Decomposition and biological colonization of native and exotic leaf litter in a Central Spain stream

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ABSTRACT

Decomposition and biological colonization of native and exotic leaf litter in a Central Spain stream

Riparian invasion by exotic trees may lead to changes in the quality of leaf-litter inputs to freshwater ecosystems. Leaf-litter inputs are especially important in headwater streams, where aquatic food webs largely depend on the organic matter provided by the terrestrial vegetation. In a headwater stream of Central Spain, North of Guadalajara Province (Tagus basin) we compared the decomposition and biological colonization of leaf litter among two exotic (*Ailanthus altissima* Mill. Swingle and *Robinia pseudoacacia* L.) and two native trees (*Fraxinus angustifolia* Vahl. and *Populus alba* L.) co-existing in riparian zones. We hypothesized a lower biological colonization of exotic leaves due to organisms have not co-evolved with the exotic trees according to the *Novel Weapons Hypothesis* (NWH) and, consequently, lower decomposition rates of exotic leaves. To test our hypothesis, litter bags with different mesh sizes (coarse (C) and fine (F)), were used to distinguish between the total and microbial decomposition. The litter bags were placed in the stream and recovered after 2, 20, 39, 62 and 82 days of incubation. After each collection, we assessed the amount of remaining litter, analysed the nitrogen (N) and phosphorous (P) in the litter, identified the macroinvertebrates and quantified the fungal biomass. Consistent with our hypothesis, we found greater fungal biomass buildup on native leaves than on exotic leaves. However, decomposition rates (k) were species-specific, being the ranking in C-bags (*A. altissima = F. angustifolia > P. alba > R. pseudoacacia*) slightly different than in F-bags (*A. altissima > F. angustifolia > P. alba > R. pseudoacacia*). The k in both the C-bags and F-bags were correlated with leaf traits, such as lignin, acid detergent fibre (ADF) and lignin:P. There was not a clear effect of the exotic species on macroinvertebrate variables, but the contribution of macroinvertebrates to litter decomposition was lower in exotic litter than in native litter, as shown by a lower ratio between k in C-bags and k in F-bags ($k_C : k_F$). Our results supported that invasive species might impact both biological colonization and decomposition rates in stream ecosystems by the addition of different quality leaf litter when compared to native vegetation. The differences in the biological colonization may be explained by the NWH, but differences in the decomposition rates were better explained by litter quality.

Key words: *Ailanthus altissima*, freshwater ecosystem, fungal biomass, invasive species, leaf quality, macroinvertebrate community, *Robinia pseudoacacia*.

RESUMEN

Descomposición y colonización biológica de hojarasca nativa y exótica en un arroyo del centro de España

*La invasión de las riberas por árboles exóticos puede provocar cambios en la calidad de las entradas de hojarasca a los ecosistemas de agua dulce, especialmente importantes en arroyos de cabecera, donde las redes tróficas dependen en gran medida de los aportes de materia orgánica proporcionada por la vegetación terrestre. En un arroyo de cabecera del Centro de España, Norte de la provincia de Guadalajara (Cuenca del Tajo), comparamos entre la descomposición y colonización biológica de la hojarasca de dos árboles exóticos (*Ailanthus altissima *Mill. Swingle y* Robinia pseudoacacia *L.) y dos nativos (*Fraxinus angustifolia *Vahl. y* Populus alba *L.), que coexisten en zonas de ribera. Hipotetizamos que habrá una menor*

*colonización biológica de las hojas exóticas debido a que los organismos del arroyo no han co-evolucionado con los árboles exóticos de acuerdo con la Hipótesis de las Nuevas Armas (NWH) y, por tanto, menores tasas de descomposición de éstas. Para testar nuestra hipótesis, se sumergieron en el arroyo bolsas de descomposición con distinta de luz de malla, gruesa (C) y fina (F) –usadas para distinguir entre descomposición total y microbiana– y se recuperaron después de 2, 20, 39, 62 y 82 días de incubación. Después de la recolección pesamos la cantidad de hojarasca remanente, analizamos el nitrógeno (N) y fósforo (P) de la hojarasca, identificamos los macroinvertebrados y cuantificamos la biomasa fúngica. De acuerdo con nuestra hipótesis, encontramos mayor biomasa fúngica acumulada en hojas nativas que en exóticas. Sin embargo, la tasa de descomposición (*k*) fue específica de la especie, siendo el ranking en las bolsas C (A. altissima = F. angustifolia > P. alba > R. pseudoacacia) ligeramente diferente al de las bolsas F (A. altissima > F. angustifolia > P. alba > R. pseudoacacia). Ambas k (en bolsas C y F) se correlacionaron con lignina, fibra ácido detergente (ADF) y lignina:P. No hubo un efecto claro de las especies exóticas sobre las variables de macroinvertebrados, pero la contribución de los macroinvertebrados a la descomposición fue menor en la hojarasca exótica que en la nativa, como muestra el menor ratio entre k en bolsas C y F (k$_C$: k$_F$). Nuestros resultados apoyan que las especies invasoras pueden impactar tanto la colonización biológica como las tasas de descomposición de la hojarasca en los ecosistemas de arroyos mediante el aporte de hojarasca de distinta calidad respecto de la vegetación nativa. Las diferencias en colonización biológica pueden ser explicadas por la NHW pero las diferencias en descomposición son mejor explicadas por la calidad de la hojarasca.*

Palabras clave: *Ailanthus altissima, ecosistemas de agua dulce, biomasa fúngica, especies invasoras, calidad de la hoja, comunidad de macroinvertebrados,* Robinia pseudoacacia.

INTRODUCTION

Litter inputs into streams from riparian areas represent the main food source for heterotrophic communities in headwater streams, where the shade cast by riparian canopies limits autochthonous primary production (Wallace *et al.*, 1997). In temperate deciduous forest, fallen leaves during autumn are the main component of the litter inputs (Abelho, 2001). When leaf litter falls into a stream, a complex decomposition process begins which involves an initial leaching of soluble compounds, followed by physical abrasion and colonization by microbes and invertebrates (Webster & Benfield, 1986). The rate of leaf decomposition depends on the local climatic conditions, water properties, biotic communities, and the quality of the leaves (Webster & Benfield, 1986). Leaf chemical composition (e.g., content of nitrogen (N), phosphorous (P), phenolic compounds, and lignin), along with physical properties (e.g., leaf toughness, leaf mass per unit of area, and density) represent the leaf quality, which is a major factor determining the decomposability of leaves (Enriquez *et al.*, 1993; Ostrofsky, 1997; Graça & Canhoto, 2006; Bottollier-Curtet *et al.*, 2011).

The quality of leaf litter may affect aquatic detritivores. For instance, leaf secondary compounds, such as polyphenols or essential oils, may delay or inhibit fungal growth (Graça *et al.*, 2002; Mathuriau & Chauvet, 2002), which in turn may affect leaf consumption by macroinvertebrates because microbial colonization (i.e., conditioning) make leaves more palatable to detritivores (Arsuffi & Suberkropp, 1989; Wright & Covich, 2005). Moreover, it was reported that aquatic macroinvertebrates preferentially consumed soft leaves with high N content and low amounts of structural carbon (Motomori *et al.*, 2001; Rincón & Martínez, 2006). The growth and survival of macroinvertebrates increased with the consumption of such high-quality leaves (Albariño & Balseiro, 2002; Going & Dudley, 2008). The density and diversity of invertebrates may be directly affected by exotic trees bearing leaves with high secondary compounds, as is the case of *Eucalyptus* spp. (Larrañaga *et al.*, 2009; Gama *et al.*, 2014). However, in the *Eucalyptus* spp. native range, the diversity of invertebrates was reduced by the introduction of the exotic *Salix* spp. (Read & Barmuta, 1999). The negative effect of eucalyptus on invertebrates in its exotic range, but not in its native range, suggested that

invertebrates in the native range were adapted to the chemicals present in *Eucalyptus* spp. leaves, which were novel for those in the exotic range. In this context, the *Novel Weapons Hypothesis* (NWH) described by Callaway & Ridenour (2004) predicts that secondary chemical compounds produced by exotic species affect microorganisms and invertebrates in their areas of introduction because these organisms are not adapted to these compounds.

Riparian ecosystems are highly vulnerable to exotic plant invasion (Hood & Naiman, 2000) due to their milder microclimatic conditions and the frequent natural and anthropogenic disturbances that create diverse niches to support invasive species (Planty-Tabacchi *et al.*, 1996; Chytrý *et al.*, 2008). Vegetation destruction by human activities additionally increases the invasibility of these systems, creating gaps that can be readily colonized by light-demanding invaders (Lindig-Cisneros & Zedler, 2001). Floodplain invasions by exotic trees may alter the quality of leaf litter inputs into streams, thus causing effects on heterotrophic communities and nutrient cycles (Bailey *et al.*, 2001; Hladyz *et al.*, 2009). Those effects will depend on the magnitude and direction of the leaf quality differences between the exotic and the native species (Hladyz *et al.*, 2009).

Ailanthus altissima Mill. Swingle (Simaroubaceae) and *Robinia pseudoacacia* L. (Fabaceae) are two aggressive invasive tree species in many regions of the world, and they usually invade riparian areas (Sanz-Elorza *et al.*, 2004; GEIB, 2006; DAISIE, 2009). *A. altissima* is native to Southeast Asia and *R. pseudoacacia* is native to the East and Central United States (Kowarik & Säumel, 2007; Cierjacks *et al.*, 2013). Some of the traits that may contribute to their invasive potential are the production of phytotoxic compounds (De Feo *et al.*, 2003; Nasir *et al.*, 2005), a profuse resprouting capacity and, in the case of *R. pseudoacacia*, the ability to fix atmospheric N_2, which is very useful in nutrient-poor soils (Rice *et al.*, 2004; Kowarik & Säumel, 2007; Cierjacks *et al.*, 2013). Both exotics are in the Atlas of exotic invasive plants in Spain (Sanz-Elorza *et al.*, 2004), and they co-occur with native riparian trees, such as *Fraxinus angustifolia* Vahl. (Oleaceae) and *Populus alba* L. (Salicaceae).

Some studies have investigated the effect of *A. altissima* and *R. pseudoacacia* on riparian soils and on lentic ecosystems of Central Spain (Castro-Díez *et al.*, 2011; Alonso *et al.*, 2010), but studies on stream ecosystems, which are highly depend on terrestrial inputs from riparian vegetation, are scarce (see Swan *et al.*, 2008). Moreover, studies about the effect of *A. altissima* and *R. pseudoacacia* on the fungal colonization of leaves are lacking, even when aquatic fungi are an important component involved in leaf decomposition (Abelho, 2001; Cornut *et al.*, 2010).

The aim of this study was to assess the impacts of *A. altissima* and *R. pseudoacacia* on key functions of a stream ecosystem in Central Spain. Specifically, we compared leaf decomposition rates, fungal and macroinvertebrate colonization, and nutrient dynamics between exotic and two common native tree species (*F. angustifolia* and *P. alba*). Because leaf quality is the main factor determining leaf decomposition through the effect on macroinvertebrates and fungi, the differences in some leaf traits among species were also explored. We hypothesized that exotic leaf litter would show lower fungal and macroinvertebrate colonization than native leaf litter, given that stream organisms have not co-evolved with the exotic tree secondary compounds (NWH; Callaway & Ridenour, 2004) and, consequently, the decomposition rates of the exotic leaf litter in the stream were expected to be lower than those of the native leaf litter.

MATERIAL AND METHODS

Study area and leaf collection

On autumn 2010, we performed the experiment at the Alboreca Stream, in the upper basin of the Henares River (Guadalajara, Spain; latitude 41°8′N, longitude 2°36′W, elevation 1056 m). The climate in the area is Mediterranean pluvi-seasonal-oceanic, with mean maximum and minimum annual temperatures of 31.5 °C and −2.1 °C, respectively, and a mean annual preci-

pitation of 601 mm (Worldwide Bioclimatic Classification System, http://www.globalbioclimatics.org). The riparian vegetation of the upper basin of the Henares River is characterized by native trees *P. alba and F. angustifolia*, with some patches invaded by the exotic tree species *A. altissima* and *R. pseudoacacia*. At the study site, the floodplain is mostly occupied by crops and *Populus x euramericana* plantations.

The wetted width, stream depth and current velocity of the study site were measured at the beginning and the end of the experiment using a metre stick and a portable velocitymeter (Series P600, Dostmann electronic GmbH, Ladytown, Ireland). The maximum and minimum water temperatures, pH, conductivity and nutrient concentrations were monitored during the leaf litter decomposition experiment on each collection date. The water temperature was measured with an outdoor maximum/minimum thermometer placed inside the stream. In the laboratory, the stream water pH and conductivity were measured with a pH meter (Crison microPH 2001) and a conductivity meter (Crison conductivity meter 524), respectively. The water nitrate, ammonium and ortho-phosphate were measured using photometric methods (Spectroquant®test).

Senescent leaves, i.e., those which detach with a gentle touch, from *R. pseudoacacia*, *A. altissima*, *F. angustifolia* and *P. alba* were collected in October 2009 from the riparian forest along the low stretch of the Henares River (near the city of Alcalá de Henares, Madrid, Spain). For each species, leaves were collected from multiple trees, pooled and air-dried at room temperature. Rachises from compound leaves (*A. altissima*, *F. angustifolia* and *R. pseudoacacia*) and petioles of *P. alba* were removed to homogenize the leaf sampling used for this experiment. Although we only used leaf laminas, we will refer to them as "leaves" throughout the paper.

Leaf litter decomposition

Air-dried leaves from each tree species were incubated in the stream using bags with two different mesh sizes: 1) fine mesh bags (F-bags) of 12×12 cm were made with 1.2 mm nylon mesh

size, which excluded macroinvertebrate access and allowed only microbial decomposition; and 2) coarse mesh bags (C-bags) of 17×17 cm were made with high density 5 mm polyethylene mesh size and allowed both microbial and macroinvertebrate processing. Each bag was filled with approximately 2 g of senescent air-dried leaves of a single species. On 12 November 2010, a total of 252 litter bags were placed in 2 stream riffles, separated 15 m from each other. Among them, 160 litter bags (4 leaf species × 2 types of litter bags × 5 collections × 4 replicates) were used to assess litter decomposition and to identify the macroinvertebrates colonizing the litter material. Seventy-two additional litter bags (4 leaf species × 2 types of litter bags × 3 collections × 3 replicates) were used for the fungal biomass estimation (see below). The remaining 20 bags were empty C-bags (5 collections × 4 replicates) that were used as a control for the macroinvertebrates that used the bags as refuge. Each replicated bag (1 per species, litter bag type and collection) was fixed in a random position to one of two chains, fastened to one of four concrete blocks. Half of these blocks were placed in each riffle. In this way, the potential effect of the riffle and the block on litter decomposition and colonization was homogeneously distributed among species, bag types and collections. Litter bags were collected after 2, 20, 39, 62, and 82 days of incubation by means of a hand net. Before the third collection, there was a sudden rise of the water flow (detected 40 km downstream 12 days before the 3rd collection by Bujalaro gauging station), which moved all of the concrete blocks from their initial position, and possibly dragged part of the invertebrates which had already colonized the litter bags. However, no litter bag or its content was lost. Three days before the third collection, the blocks were placed back in their initial positions to keep the initial site conditions. After each collection, samples were stored in polyethylene zipper bags, transported and kept cold (5 °C) until processing. Once in the laboratory, the litter bags were gently rinsed with tap water over a 250 μm sieve to separate the macroinvertebrates from the leaves. Macroinvertebrates were preserved in a 3 % formaldehyde solution until they were iden-

tified to the family level using the method in Tachet *et al*. (2003). Macroinvertebrates were additionally classified into the following functional feeding groups (FFG): shredders (SH), collector-gatherers (CG), collector-filterers (CF), scrapers (SC) and predators (PD), according to Merritt & Cummins (1996) and Tachet *et al*. (2003). The leaf mass remaining (LMR) in each bag was oven-dried ($\geq$ 48 h at 60 °C) and weighed.

Leaf traits

We assessed the N and P concentrations in three 2 g-replicates of the initial senescent leaves and the total remaining leaf mass in each collection and species from C-bags. Samples were oven-dried ($\geq$ 48 h at 60 °C) to correct for water content and ground with a Culatti mill to a particle size < 0.5 mm. The N and P concentrations were determined with a segmented flux auto-analyser (Skalar San^{++}) after a digestion with H_2SO_4 and $Cu-KSO_4$ (Nelson & Sommers, 1973). Acid detergent fibre (ADF), lignin and total phenolic compounds (TPC) were analysed only in the non-decomposed leaves. ADF and lignin concentrations were determined using the filter bag technique (FBT), with an Ankom Automated Fibre Analyser A2000, based on the analytical method of Goering & Van Soest (1970). The extraction procedure to determine the TPC was performed in duplicate as follows. Approximately 200 mg of freeze-dried and ground leaves were extracted twice in 5.0 ml of 50:50 (v/v) methanol:water for 30 min in an ultrasound bath. The mixture was centrifuged and filtered twice through filter paper, and the filtrates were mixed and stored at −20 °C until analysis (Waterman & Mole, 1994). The TPC concentration was assessed by the colorimetric Prussian-blue method using gallic acid as the
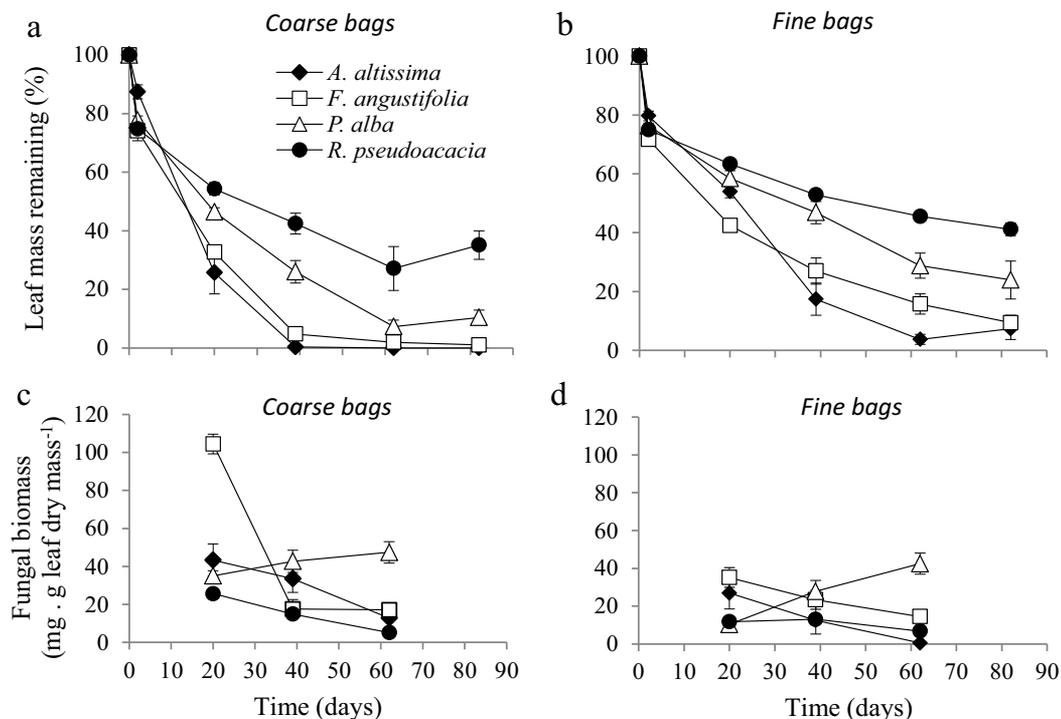
Figure 1. The mean (± SE) leaf mass remaining (a and b) and fungal biomass (c and d) in the coarse (a and c) and fine (b and d) litter bags for each species over the study period. Black symbols indicate exotic species and white symbols indicate native species. *Media (± EE) de la masa de hoja remanente (a y b) y de la biomasa fúngica (c y d) en bolsas de descomposición de luz de malla gruesa (a y c) y fina (b y d) para cada especie en el periodo de estudio. Los símbolos negros indican especies exóticas y los blancos especies nativas.*

Table 1. Mean values (± SE) for initial leaf traits of exotic and native species. N = Nitrogen; P = Phosphorus; TPC = Total phenolic compounds; and ADF = Acid Detergent Fibre. Different letters in a row indicate significant differences among species for each variable (ANOVA test with Tukey HSD, $p < 0.05$), and they are alphabetically ordered from the highest to the lowest value. *Valores medios (± EE) de las características iniciales de las hojas de las especies exóticas y nativas. N = Nitrógeno; P = Fósforo; TPC = Compuestos fenólicos totales; ADF = Fibra Ácido Detergente. Diferentes letras en una fila indican diferencias significativas entre especies para cada variable (ANOVA test con Tukey HSD, p < 0.05 y están ordenadas alfabéticamente de mayor a menor valor.*

	Exotics		Natives	
Leaf traits	*A. altissima*	*R. pseudoacacia*	*P. alba*	*F. angustifolia*
N (%)	1.51 ± 0.01 [b]	1.75 ± 0.01 [a]	0.93 ± 0.02 [d]	1.00 ± 0.00 [c]
P (%)	0.10 ± 0.01 [ab]	0.07 ± 0.00 [c]	0.07 ± 0.00 [bc]	0.15 ± 0.02 [a]
Lignin (%)	18.1 ± 0.7 [b]	29.5 ± 1.5 [a]	32.6 ± 1.8 [a]	22.2 ± 0.5 [b]
ADF (%)	23.1 ± 1.3 [c]	31.7 ± 0.3 [b]	36.1 ± 1.1 [a]	26.0 ± 0.6 [c]
TPC (%)	2.3 ± 0.4 [a]	2.7 ± 0.0 [a]	2.2 ± 0.1 [a]	1.1 ± 0.0 [b]
N:P	14.9 ± 1.5 [b]	26.3 ± 0.8 [a]	13.2 ± 0.5 [b]	6.8 ± 0.9 [c]
Lignin:N	11.9 ± 0.4 [c]	16.9 ± 0.9 [b,c]	35.2 ± 2.3 [a]	22.2 ± 0.6 [b]
Lignin:P	178.9 ± 23.8 [b]	444.9 ± 34.9 [a]	465.5 ± 41.4 [a]	149.8 ± 16.4 [b]

standard (Graham, 1992). We calculated the litter quality indices N:P, lignin:N and lignin:P ratios because they were previously related to decomposition rates and dynamics (Hladyz *et al*., 2009).

Fungal biomass determination

The fungal biomass in the litter samples, collected after 20, 39 and 62 days in the stream, was estimated from the concentration of ergosterol, a specific component of fungal cell membranes (Ekblad *et al*., 1998), following the methodology of Gessner (2005). In brief, freeze-dried leaf samples (approximately 0.10 g) were placed in tubes with 10 ml of 0.14 M of potassium hydroxide (KOH) in methanol. The mixture was incubated at 80 °C during 30 min in a Block Heater (Stuart Scientific). After being cooled, the

extract was acidified with 5 ml of 0.75 M of hydrochloric acid (HCl) and transferred into a SPE cartridge (Waters Sep-Pack Vac RC, 500 mg, tC18) pre-conditioned with an acidified extract solution (0.14 M KOH in methanol: 0.75 M HCl; 6:1 v/v). After the sample addition, the column was washed with 2.5 ml of 0.4 M KOH in 60 % methanol (v/v) and dried under a stream of air for approximately 60 min. Ergosterol was eluted into HPLC vials with 1.6 ml of isopropanol. The final separation of ergosterol from the matrix lipids was performed by High Performance Liquid Chromatography (HPLC) with a Waters 2690 system with a Sunfire C18 Waters column (5 μm; 5.6 × 250 mm), at 33 °C. The mobile phase was methanol (100 %) at a flow rate of 1.4 ml/min. Ergosterol was detected at 282 nm with a Photodiode Array Detector (PDA) Waters 2996.

Table 2. Decomposition rates (k) (Mean ± 95 % Confidence interval) in coarse (k_C) and fine (k_F) litter bags, the $k_C : k_F$ ratio of the four species; and the R^2 coefficient of each regression line fitted to calculate k. Different letters in a column indicate significant differences among species ($p < 0.05$, overlap test). For each species, significant differences between k_C and k_F ($p < 0.05$, overlap test) are indicated by an asterisk. *Tasas de descomposición* (k) *(Media ± Intervalo de confianza al 95 %) en bolsas de luz de malla gruesa* (k$_C$) *y fina* (k$_F$), *el ratio* k$_C$: k$_F$ *de las cuatro especies y el coeficiente* R^2 *de cada línea de regresión ajustada para calcular* k. *Diferentes letras en una columna indican diferencias significativas entre especies* (p < 0.05; test de solapamiento). *Para cada especie diferencias significativas entre* k$_C$ *y* k$_F$ (p < 0.05; test de solapamiento) *están indicadas por medio de un asterisco.*

	Decomposition rate				$k_C : k_F$
Species	$-k_C$ (Mean (95 % CI))	R^2	$-k_F$ (Mean (95 % CI))	R^2	
A. altissima	0.080 (0.062-0.098) [a]	0.87	0.053 (0.040-0.066) [a]	0.84	1.51
R. pseudoacacia *	0.017 (0.012-0.022) [d]	0.80	0.008 (0.007-0.009) [d]	0.95	2.07
P. alba *	0.040 (0.029-0.051) [c]	0.82	0.016 (0.012-0.020) [c]	0.84	2.50
F. angustifolia *	0.069 (0.058-0.081) [a]	0.93	0.028 (0.022-0.034) [b]	0.89	2.43

The fungal biomass concentration was estimated using the conversion factor of 5.5 mg ergosterol per gram of fungal dry mass (Gessner & Chauvet, 1993).

Statistical analysis

An analysis of covariance (ANCOVA) was performed, using time as a covariate, to assess the effect of the two factors (litter bag and species) on the percentage of leaf mass remaining (% LMR), as a dependent variable. The % LMR was transformed ($\ln(x+1)$) to meet the linearity assumption for ANCOVA, and it was fitted with time to calculate the decomposition rates (k) in C-bags and F-bags (k_C and k_F, respectively). We considered that two species differed in k when their 95 % confident intervals did not overlap. The ratio $k_C : k_F$ was considered as a proxy measurement of the effect of macroinvertebrates on leaf litter decomposition, although the physical abrasion by water flow was also a factor that affected litter decomposition and acted in a greater extent in C-bags than in F-bags (Hladyz et al., 2009). Therefore, changes in the $k_C : k_F$ ratio across species implied shifts in the relative contribution of microorganisms and shredders to litter decomposition (Gessner & Chauvet, 2002). We did not use the last collection (82 days) in the analysis because there were no remains of *A. altissima* and *F. angustifolia* leaves and increments in % LMR were noticed in *R. pseudoacacia* and *P. alba*, likely due to $CaCO_3$ precipitation (Casas & Gessner, 1999). Differences among species in the initial leaf traits were assessed by one-way analysis of variance (ANOVA) followed by a Tukey Honestly Significant Difference (HSD) test. To meet the ANOVA assumptions, the P concentration was Ln transformed. Pearson correlations between leaf traits and k were performed to determine which traits controlled leaf decomposition. The differences in the N and P concentrations of the decomposed leaves among species and time were assessed by a two-way ANOVA (species × time), followed by a Tukey HSD test. The differences in the fungal biomass were assessed by a three-way ANOVA (species × litter bag × time), followed by a Tukey HSD test.

The differences in the total density (number of individuals per litter bag) and richness (number of macroinvertebrate families per litter bag) were assessed using a two-way ANOVA (species × time). To meet the ANOVA assumptions, the density of macroinvertebrate families were log-transformed. A generalized linear model (GLM, Poisson distribution, log link) was used to evaluate the differences in the relative abundance of shredders across species and time. These analyses included the control (empty bags) as a level of the factor species.

To search for patterns among the macroinvertebrate communities colonizing the different leaf species, a two-dimensional non-metric multidimensional scaling ordination (NMDS) (McCune & Grace, 2002) was performed on the mean relative abundance of the macroinvertebrate families (number of individuals of a family per total number of individuals in litter bag). Macroinvertebrate families that occurred in less than 5 % of the C-bags (i.e., 26 out of 42 families) were omitted to reduce the effect of rare taxa on the ordination (McCune & Grace, 2002). The mean relative abundances of the used families per litter species and collection are available in the Supplementary information (Table S1 at www.limnetica.net/internet). The sample positions on the NMDS biplot were calculated by Euclidean distances from the original data. Fifteen iterations were run to achieve a stress value of 0.15, which was considered satisfactory in Community Ecology (McCune & Grace, 2002). Pearson correlations between the mean relative abundance of each macroinvertebrate family and each axis of NMDs were performed to assess which macroinvertebrate families had further weight on the ordination. Families with $R^2 > 0.6$ and a significant correlation ($p < 0.05$) were considered to be the most influential on the ordination. Permutational multivariate analysis of variance (perMANOVA), using Bray-Curtis distance matrices, was applied to the relative abundance of macroinvertebrate families used in the NMDS to assess the differences across species and time in the macroinvertebrate community structure. R package 2.14.0 (R Development Core Team, 2011) was used for NMDS ordina-

Table 3. The results of a two-way ANOVA (species and time) for nitrogen (N) and phosphorous (P) concentration and a three-way ANOVA (species, time and litter bag) for fungal biomass. *Resultados del ANOVA de dos vías (especie y tiempo) para la concentración de nitrógeno (N) y fósforo (P) y el ANOVA de tres vías (especie, tiempo y bolsa de descomposición) para la biomasa fúngica.*

Source of variability	df	F	p
N (%)			
Species	3	84.8	**<0.0001**
Time	4	72.7	**<0.0001**
Species × time	12	9.2	**<0.0001**
P (%)			
Species	3	76.7	**<0.0001**
Time	4	11.4	**<0.0001**
Species × time	12	4.6	**<0.0001**
Fungal biomass			
(mg g dry mass^{-1})			
Species	3	20.2	**<0.0001**
Time	2	19.2	**<0.0001**
Litter bag	1	33.5	**<0.0001**
Species × time	6	17.3	**<0.0001**
Species × litter bag	3	2.3	0.0922
Time × litter bag	2	2.7	0.0711
Species × time × litter bag	6	4.7	**0.0007**

tion (isoMDS algorithm in R) and perMANOVA (Adonis algorithm in R) analyses, while JMP, Version 7 (SAS Institute Inc., Cary, NC, 1989-2007), was used for the rest of the analyses.

RESULTS

Stream characteristics

The mean ± SE properties of the study stream were as follows: stream wetted width = 2.23 ± 0.11 m ($n = 21$); depth = 16 ± 1 cm ($n = 157$); current velocity = 0.25 ± 0.02 m/s ($n = 157$); water conductivity = 607.4 ± 18.9 μs/cm ($n = 5$); pH = 8.05 ± 0.04 ($n = 5$); water minimum and maximum temperatures = 5 °C and 13 °C, respectively; and nutrient concentrations = 2.9 ± 0.5 mg NO_3-N l^{-1}, 0.17 ± 0.03 mg NH_4-N l^{-1}, and 0.06 ± 0.01 mg PO_4-P l^{-1} ($n = 5$). Therefore, our stream was P limited (N:P > 40).

Leaf traits

A. altissima leaves showed relatively low lignin and ADF concentration and high N, P and TPC concentrations (Table 1). By contrast, *R. pseudoacacia* leaves showed relatively low P, the highest N and relatively high concentrations of lignin, ADF and TPC. *F. angustifolia* leaves showed similar properties to *A. altissima*, except for the lower N and TPC. *P. alba* leaves exhibited high lignin, ADF and TPC concentrations and low N and P.

Leaf litter decomposition

The ranking of litter species by k_C (in the presence of macroinvertebrates) was: *A. altissima* = *F. angustifolia* > *P. alba* > *R. pseudoacacia*, while the ranking by k_F (in absence of macroinvertebrates) was: *A. altissima* > *F. angustifolia* > *P. alba* > *R. pseudoacacia* (Table 2; Figs. 1a-b). All of the species, except *A. altissima*, showed significantly greater k_C than k_F (Table 2). The interaction terms (species × time) and (litter bag × time) were highly significant (ANCOVA; $F_{3,121} = 49.4$, and $F_{1,123} = 56.1$, respectively; $p < 0.0001$), indicating that both factors (species and litter bag) had significant effects on the decomposition rates (Figs. 1a-b). In addition, the interaction term (species × litter bag × time) was also significant (ANCOVA, $F_{3,124} = 3.67$, $p = 0.014$), indicating that the effect of the litter bag in the decomposition rates was different among species. The contribution of macroinvertebrates to decomposition (ratio $k_C : k_F$) was greater in native than in exotic leaf litters (Table 2). The k_C was negatively correlated with lignin (%), ADF (%), N:P ratio and lignin:P (Pearson correlation; $r = -0.82$, $r = -0.77$, $r = -0.73$ and $r = -0.86$, respectively; $p < 0.05$). The k_C was positively correlated with P (%) (Pearson correlation; $r = 0.66$; $p < 0.05$). The k_F was negatively correlated with lignin (%), ADF (%) and lignin:P (Pearson correlation; $r = -0.86$, $r = -0.81$ and $r = -0.75$, respectively; $p < 0.05$), but no significant correlations were found between decomposition rates and the rest of leaf traits considered (N, TPC, and lignin:N).

Fungal biomass

Fungal biomass buildup on the decomposing leaves was affected by species, time, litter bag (C and F) and most of the interactions (Table 3). Native leaf litter had a higher fungal biomass than exotic leaf litter (ANOVA, Tukey HSD, $p < 0.05$; Figs. 1c-d). In the presence of macroinvertebrates (C-bags), the differences in the fungal biomass between exotic and native leaves were mainly due to the greater value in the native *F. angustifolia* at 20 days and the greater value in *P. alba* than in *R. pseudoacacia* at 62 days (ANOVA, Tukey HSD, $p < 0.05$; Fig. 1c). However, in the absence of macroinvertebrates (F-bags), both exotics showed a lower fungal biomass than natives at 39 days and 62 days (ANOVA, Tukey HSD, $p < 0.05$; Fig. 1d). In F-bags, the fungal biomass was lower than in C-bags, but only significant in the case of *F. angustifolia* (ANOVA, Tukey HSD, $p < 0.05$; Figs. 1c-d). The fungal biomass did not vary significantly throughout the study for the two exotic species in any bag type (C and F) (ANOVA, Tukey HSD, $p > 0.05$; Figs. 1c-d), but peaks were observed for the two natives at 20 days (*F. angustifolia*) and at 62 days (*P. alba*) (ANOVA, Tukey HSD, $p < 0.05$; Figs. 1c-d).

Nutrient dynamics

Litter N and P concentrations were influenced by species, time and the interaction between them (Table 3). Leaves of *R. pseudoacacia* showed the highest N and lowest P concentrations throughout the experiment (ANOVA, Tukey HSD, $p < 0.05$; Figs. 2a-b). N concentration increased in the second collection (20 days) in all of the species and then decreased in *A. altissima* and *F. angustifolia*. The N concentration kept increasing in *P. alba* and remained unchanged in *R. pseudoacacia* (ANOVA, Tukey HSD, $p < 0.05$; Fig. 2a). In the first collection (day 2), the P concentration decreased and then increased over time in *P. alba* leaves, but no variation was observed for the other leaf species (ANOVA, Tukey HSD, Fig. 2b).

Macroinvertebrate colonization

The most abundant macroinvertebrate families in the study in the NE bags were: Chironomidae (Diptera), Gammaridae (Amphipoda), Bithyniidae (Mollusca), Hydropsychidae (Trichoptera), Baetidae (Ephemeroptera) and Simuliidae (Diptera) that made up 28 %, 23 %, 12 %, 10 %, 8 % and 5 %, respectively, of the total macroinvertebrate abundance. The total macroinvertebrate density and family richness did not vary across species (ANOVA, $F_{4,84} = 1.75$, $F_{4,84} = 1.61$, respectively, $p > 0.05$) or time ($F_{4,84} = 1.52$, $F_{4,84} = 2.04$, respectively, $p > 0.05$) but were affected by its interaction ($F_{16,84} = 4.67$, $F_{16,84} = 3.07$, respectively, $p < 0.05$; Figs. 3a-b). Therefore, the density and richness of the macroinvertebrate families varied in different ways over
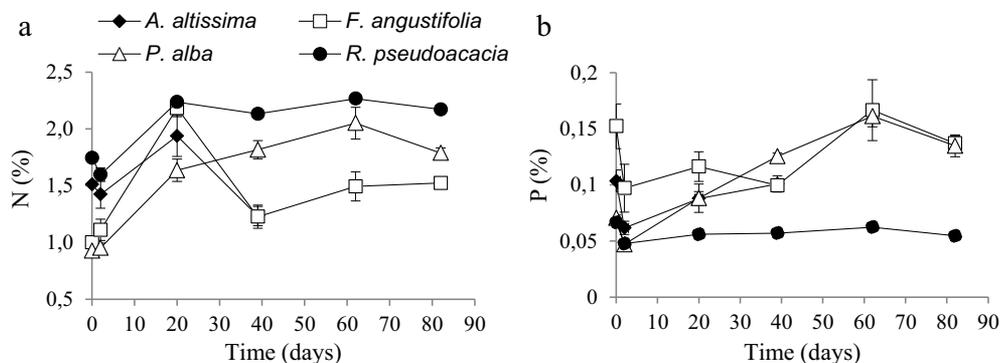
Figure 2. The mean (± SE) leaf N (a) and P (b) concentrations for each species over the study period. Black symbols indicate exotic species and white symbols indicate native species. *Media (± EE) de las concentraciones de N (a) y P (b) en las hojas para cada especie en el periodo de estudio. Los símbolos negros indican especies exóticas y los blancos especies nativas.*

a

b
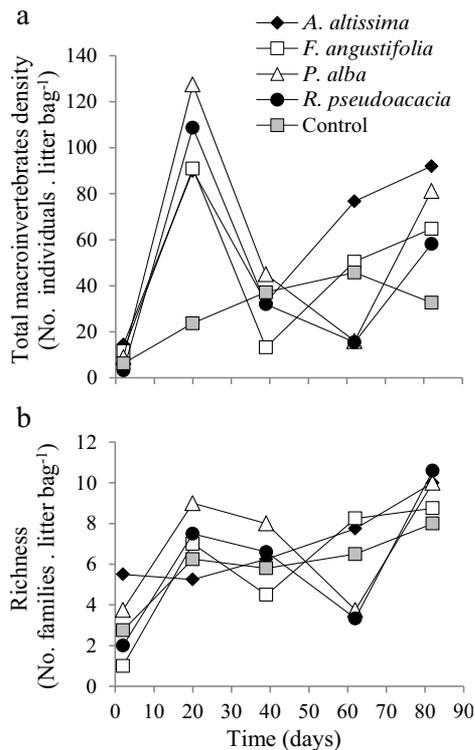
Figure 3. The mean density (a) and richness (b) of macroinvertebrates for each species over the study period. Black symbols indicate exotic species, white symbols indicate native species and grey symbols indicate empty bags (control). SE is not shown for better clarity. *Media de la densidad (a) y riqueza (b) de macroinvertebrados para cada especie a lo largo del periodo de estudio. Los símbolos negros indican especies exóticas, los blancos especies nativas y los grises bolsas vacías (controles). EE no se muestra para una mejor claridad.*

time. The macroinvertebrate density drastically dropped in the third collection (Fig. 3a), likely due to the sudden rise of flow mentioned above, which may have dragged many of the macroinvertebrates that had already colonized the litter bags. Subsequently, the macroinvertebrate density increased in the leaf litters with faster decomposition (*A. altissima* and *F. angustifolia)*, but decreased in those with slower decomposition (*P. alba* and *R. pseudoacacia*) (Fig. 3a). A similar pattern appeared in family richness, which increased in *A. altissima* and *F. angustifolia* and decreased in *P. alba* and *R. pseudoacacia* from 39 days to 62 days (Fig. 3b). However, the relative abundance of shredders did not differ across species, time or its interaction (GLM, $\chi^2 = 1.12$, $\chi^2 = 1.39$, $\chi^2 = 3.85$, respectively, $p > 0.05$).

The first axis of the NMDS was negatively correlated with the abundance of Gammaridae (Amphipoda) and positively correlated with the abundance of Chironomidae (Diptera) (Fig. 4b). Axis 2 was negatively correlated with the abundance of Bithyniidae (Mollusca) and Chironomidae and positively correlated with the abundance of Simuliidae (Diptera), Perlodidae (Plecoptera) and Hydropsychidae (Trichoptera) (Fig. 4b). The NMDS biplots showed that macroinvertebrates from the four leaf species appeared close to each other in the first three collections, except those of *P. alba*, which appeared separated from the other species in the first collection (Fig. 4a). On the contrary, samples of the two last collections were scattered throughout the ordination axes. In the fourth collection, after 62 days in the stream, samples corresponding to native leaves appeared clustered and separated from those corresponding to the exotic leaves. In the fifth collection, after 82 days in the stream, *P. alba* and *R. pseudoacacia* samples appeared clustered and far from *A. altissima* and *F. angustifolia* along axis 1 of the NMDS. However, the perMANOVA showed that only time significantly affected the composition of macroinvertebrate families ($F_{4,84} = 4.09$, $p < 0.05$).

DISCUSSION

Riparian invasion by exotic trees implies that streams receive inputs of leaves with properties that may widely differ from the native leaves, which may alter the structure and function of aquatic communities (Webster *et al*., 1990). Our study showed that 1) fungal colonization was lower in exotic than in the native leaf litter and 2) the contribution of macroinvertebrates to leaf decomposition (as reflected by $k_C : k_F$) was more important in native than in exotic leaves, although no differences were found in the macroinvertebrate density. In accordance with the NWH, fungi may better colonize the litter from those species they have co-evolved with, and macroinvertebrates may lower the effect on exotic leaves because they are not pre-adapted to their secondary compounds (Callaway & Ridenour, 2004;

a

b

	Pearson correlation test			
	Axis 1		Axis 2	
	r	p	r	p
Chironomidae	0.81	**< 0.0001**	-0.64	**0.0022**
Gammaridae	-0.81	**< 0.0001**		
Simuliidae			0.73	**0.0002**
Hydropsichidae			0.8	**< 0.0001**
Bithynidae			-0.66	**0.0016**
Perlodidae			0.62	**0.0036**
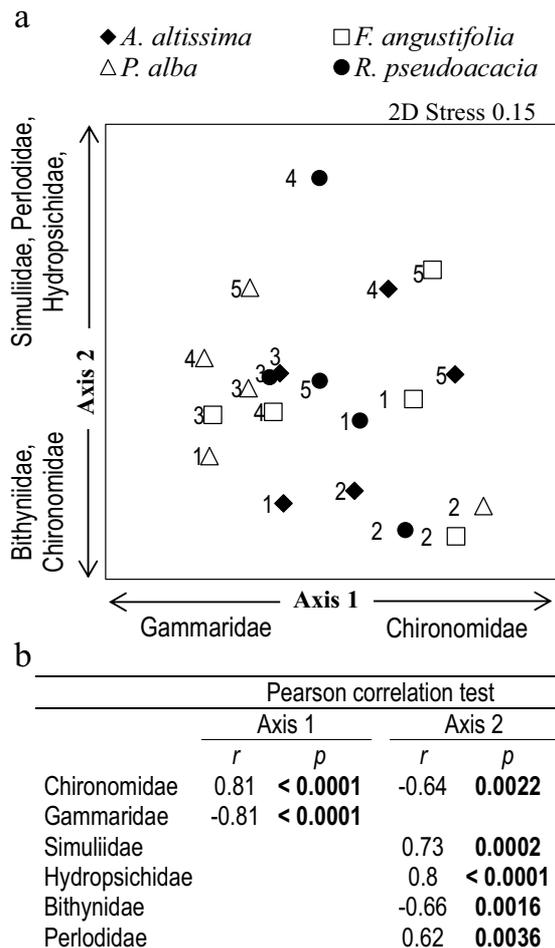
Figure 4. (a) Two dimensional Non Metric Multidimensional Scaling (NMDS) for C-bags using the mean relative abundances of macroinvertebrate families per leaf species and collection time. The symbols represent different species. Black symbols indicate exotic species and white symbols indicate native species. The numbers next to the symbols designate collection times (1 = 2 days, 2 = 20 days, 3 = 39 days, 4 = 62 days, 5 = 82 days). (b) Pearson correlation test between mean relative abundance of macroinvertebrate families and NMDS axis. Significant correlations (p < 0.05) are indicated in bold. *(a) Escalamiento Multidimensional No Métrico (NMDS) en dos dimensiones para las bolsas de descomposición C usando la media de las abundancias relativas de las familias de macroinvertebrados por especie de hoja y tiempo de colecta. Los símbolos representan diferentes especies. Símbolos en negro indican especies exóticas y en blanco especies nativas. Los números junto a los símbolos indican tiempos de colecta (1 = 2 días, 2 = 20 días, 3 = 39 días, 4 = 62 días, 5 = 82 días). (b) Test de correlación de Pearson entre la media de las abundancias relativas de las familias de macroinvertebrados y los ejes del NMDS. Las diferencias significativas (*p < 0.05) están indicadas en negrita.*

Gama *et al*., 2014). Moreover, the greater fungal colonization may also explain the greater ef-

fect of macroinvertebrates on native leaves than on exotic leaves because macroinvertebrates usually prefer more leaves preconditioned by microorganisms (Wright & Covich, 2005). Finally, the $k_C : k_F$ ratio, indicating the balance in the contribution of microorganisms and detritivorous macroinvertebrates, accounted for the responses to different leaf litter quality (Gessner & Chauvet, 2002). This balance changed in the presence of *A. altissima* and *R. pseudoacacia* leaf litter, which differed in quality from that of native species.

Given that fungi and macroinvertebrates are important agents involved in leaf litter decomposition (Abelho, 2001; Cornut *et al*., 2010) and they affected the exotic litter less than the native litter, slower decomposition rates (k) would also be expected for exotic leaves rather than for native leaves. However, physical-chemical differences across leaf species better explained the differences in k than their origin (exotic vs. native), in accordance with previous studies (Hladyz *et al*., 2009; Bottollier-Curtet *et al*., 2011; Casas *et al*., 2013). The ranking of k in the presence of macroinvertebrates was *A. altissima = F. angustifolia > P. alba > R. pseudoacacia*, slightly different from the ranking found in their absence (*A. altissima > F. angustifolia > P. alba > R. pseudoacacia*). Lignin, ADF and lignin:P were good predictors of k in the presence of macroinvertebrates (k_C) and in their absence (k_F), as previously reported (Ostrofsky, 1997; Hladyz *et al.,* 2009). Moreover, the leaf P and N:P ratio were also important in determining k_C, according to Hladyz *et al*. (2009). Physical abrasion by stream water flow is also an important factor determining leaf decomposition (Abelho, 2001). For instance, the fibres, such as lignin or ADF, increased the resistance of leaf lamina to fracture, which in turn determined the lower effect of water flow on k (Choong *et al.,* 1992). Consequently, *A. altissima* and *F. angustifolia* leaves, which presented the lowest ADF and lignin concentrations, decomposed faster than leaves of the other species.

According to NWH, the presence of secondary compounds in the leaves of both exotics, such as robinetin, myricetin and quercetin in *R. pseudoacacia* (Nasir *et al.,* 2005), and ailantho-

ne, chaparrine, and ailanthinol B in *A. altissima* (De Feo *et al.,* 2003), may explain their poor fungal colonization. Phytotoxic compounds of other species were found to inhibit microbiological activity in soils (Llinares *et al*., 1993), and essential oils from *Eucalyptus globulus* may also delay fungal growth (Graça *et al.,* 2002). Fungal biomass was reported as a good predictor of *k* (Hladyz *et al.,* 2009) and could explain why the native litter from *P. alba* decomposed faster than that from the exotic *R. pseudoacacia,* even when they had similar fibre concentrations. The high fungal biomass found in *F. angustifolia* leaves at 20 days of stream immersion was probably due to its low phenolic concentration because phenolic compounds may depress fungal growth (Mathuriau & Chauvet, 2002). Moreover, fungi could take nutrients from the water column to compensate for the effect of low-quality litter in streams with high nutrient levels (Gulis & Suberkropp, 2003). Therefore, given that our stream was P-limited (N:P > 40), the high P content of *F. angustifolia* leaves may additionally contribute to a high colonization by fungi. Accordingly, we found that the P concentration in leaves had a positive effect on decomposition rates. *F. angustifolia* leaves decomposed slower than *A. altissima* in the absence (F-bags), but not in the presence (C-bags), of macroinvertebrates, probably because the fungal colonization in *F. angustifolia* leaves was greater in F-bags than in C-bags. The overall lower fungal biomass found in F-bags compared with C-bags could be explained by two non-exclusive arguments. First, given that aquatic hyphomycetes (the main microbial decomposers in streams; Gulis & Suberkropp, 2003) are sensitive to oxygen availability (Medeiros *et al.,* 2009), poor oxygen diffusion into F-bags, due to the smaller mesh size, may have hampered the colonization of litter by this group of microbes. Second, macroinvertebrates may enhance fungal development on leaf litter, as their faeces represent a source of food for fungi (Villanueva *et al*., 2012).

Although we found similar or greater k of *A. altissima* than that of *F. angustifolia* in the stream, Alonso *et al*. (2010) found the contrary in a man-made lake. This suggested a higher vulnerability of *A. altissima* leaves to physical abrasion by the stream water flow. In fact, *A. altissima* presented soft leaves (Swan *et al*., 2008), which acquired a gelatinous consistency during decomposition (personal observation), and the k of soft leaves were enhanced by the water flow (Abelho, 2001). In addition, the absence of shredders in the man-made lake made microbial decomposition more relevant (Alonso *et al*., 2010), which may favour the decomposition of *F. angustifolia* over *A. altissima*, since, as reported above, the native species may buildup greater fungal biomass than the exotic species.

Decomposition rates may vary across streams with different characteristics, such as temperature, nutrient levels, macroinvertebrates or riparian vegetation (Grubbs & Cummins, 1994; Pérez *et al*., 2011; Menéndez *et al*., 2011). For instance, Casas *et al*., (2013) found that *k* was greater in streams receiving lower quality litter than in streams receiving higher quality litter from the riparian vegetation. However, the differences in k among leaves from *Alnus glutinosa* L., *Quercus pyrenaica* Wild, *Pinus radiata* D. Don., and *Platanus hispanica* Mill. ex Münchh. remained unaltered across streams, as the quality of litter was the most important factor determining these differences (Casas *et al*., 2013). This suggested that in our study, the differences found in k among species would be extensible to other streams. Decomposition rates may also vary among seasons due to changes in water temperature or in the structure of the macroinvertebrate community (Pérez *et al*., 2011). The four leaf species used in our study have their leaf litterfall peaks in autumn, which makes the choice of that season more relevant under an ecological perspective.

Increases in the N litter concentration over the decomposition process could be attributed to microorganism's N uptake from the water column and subsequent immobilization in form of the microbial biomass (Kuehn *et al*., 2000). Additionally, a faster loss of N-rich than N-poor leaf litter compounds may occur during decomposition. The greater N concentration observed in *R. pseudoacacia* litter throughout the experiment suggested that the organic N concentration

may increase in the stream upon invasion by this species, as reported for the N-fixing species *Elaeagnus angustifolia* (Mineau *et al.*, 2011). Furthermore, nitrate accumulation was reported in wetlands adjacent to *R. pseudoacacia* stands (Williard *et al.*, 2005). Therefore, if *R. pseudoacacia* invaded riparian zones lacking native N-fixing trees, stream N would increase.

We found no differences in the relative abundance of shredders, total density and the richness of macroinvertebrates across the leaves of different species, suggesting that macroinvertebrates were able to use both exotic and native leaves, either as refuge or food, as reported by other studies (Albariño & Balseiro, 2002). The density of macroinvertebrates drastically dropped after the hydropeaking event that occurred in the middle of the study period. Similarly, in a regulated stream, Mendoza-Lera *et al.* (2010) found that after water discharges, the density and biomass of macroinvertebrates decreased, which slowed down decomposition. This suggested that hydropeaking events may affect, to a greater extent, leaf species whose decomposition is mainly determined by macroinvertebrates. This fact could be important in regulated streams, which are frequently impacted by hydropeakings.

The macroinvertebrate community varied highly during the study period across collections but not across species. However, we could note some different trends at the final stages of decomposition, likely due to different leaf chemistry, fungal leaf preconditioning and/or availability of remaining material (Casas & Gessner, 1999). For instance, the weight of the Chironomidae (collector-gatherer) family, which feed on depositional fine particulate organic matter (FPOM), was higher in litter bags with little or no remaining mass (*A. altissima* at 62 days and 82 days and *F. angustifolia* at 82 days). At 62 days in the stream, Gammaridae (shredder) were dominant in native leaves, contrasting with the high weight of Simuliidae, Perlodidae and Hydropsychidae in *R. pseudoacacia* leaves. The most abundant families found in *R. pseudoacacia* litter bags at 62 days corresponded to functional feeding groups which did not feed directly on leaves (i.e., collector-filterers and predators),

suggesting that they used *R. pseudoacacia* leaves as a refuge or as a source of prey. By contrast, the Gammaridae family preferentially consumed fungal pre-conditioned leaves (Graça *et al.*, 1993; Foucreau *et al.*, 2013), explaining the high weight of Gammaridae in *P. alba* leaves, which were more colonized by fungi than *R. pseudoacacia*. This result may also account for the co-evolution of Gammaridae with the associated fungal species in *P. alba* leaves (according to NWH) because fungal species composition in the leaves determined the feeding activity of a variety of shredders (Arsuffi & Suberkropp, 1989). Therefore, the Gammaridae family may be negatively affected if *P. alba* was eventually displaced by exotic trees.

Because the differences in leaf decomposition among species were species-specific, the consequences of the exotic species on k will depend on the prevailing native species of the invaded ecosystem. For instance, a greater impact on *k* can be expected if *A. altissima* replaces vegetation dominated by *P. alba* (decomposition would be accelerated) and if *R. pseudoacacia* replaces vegetation dominated by *F. angustifolia* (decomposition would slow down). Given the fast decomposition of *A. altissima*, a riparian invasion by this species may reduce the time of food availability to shredder macroinvertebrates (Wallace *et al.*, 1999), affecting their life cycle, particularly in those species where the major growth period occurs in late autumn and winter (e.g., many Trichoptera) (Anderson & Cummins, 1979).

In conclusion, this study showed the potential impact of the displacement of native vegetation (characterized by *P. alba* and/or *F. angustifolia*) by the exotics *A. altissima* and/or *R. pseudoacacia* in a headwater stream ecosystem. The exotic *A. altissima* may increase decomposition rates, while *R. pseudoacacia* would have the reverse effect. The intensity of these impacts depends on the native vegetation that they replace. Leaf species and their chemical characteristics (mainly fibre concentration and phosphorous) controlled the within-stream leaf decomposition. Moreover, leaves of both exotic species were less colonized by fungi, which may affect macroinvertebrates that feed on preconditioned

leaves. The Gammaridae family could be one of the most affected because they tended to have a higher weight on the macroinvertebrate community, colonizing more leaves of *P. alba* than other species. The NWH could explain the lower fungal colonization and lower effect of macroinvertebrates on litter decomposition in exotic species than in native species, while the contrasting decomposition rates among species could mainly be explained by the litter quality.

ACKNOWLEDGEMENTS

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