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RESEARCH ARTICLE

Decomposition of leaf litter mixtures in streams: effects of component litter species and current velocity

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Abstract

The effects of mixing different leaf litter species on litter decomposition in streams have received considerable attention in recent years. However, contrasting results have been reported and the mechanisms behind the effects of litter diversity have been poorly examined. We compared the decomposition rates and associated fungi for two contrasting litter species, when incubated individually and in mixture, at two different current velocities. Coarse-mesh bags with alder litter individually, oak litter individually and with a mixture of both were incubated in a forest headwater stream over 32 days, under fast or slow current velocities. We determined litter decomposition rates, microbial oxygen consumption rates, and aquatic hyphomycete sporulation rates, species richness and community composition; litter species in the mixture were processed individually. Our results provided weak evidences for diversity effects on leaf litter decomposition. Generally, litter decomposition was unaffected by mixing contrasting litter species, with litter species in the mixture decomposing at the same rate as when incubated individually at both current velocities. The same pattern was observed for microbial variables. Decomposition rates and microbial colonization and activity depended primarily on the traits of the target litter species and were not affected by those of the companion species. However, litter-mixing effects were detected on oak litter at late decomposition stages under fast current velocity conditions, suggesting that both current velocity and the incubation time might influence diversity effects on litter decomposition in streams. This finding contributes to explain the lack of litter-mixing effects reported previously by many studies.

Keywords Aquatic decomposers · Aquatic hyphomycetes · Flow velocity · Decomposition rates · Leaf litter · Diversity effects

Introduction

Forest streams are often densely shaded by the riparian vegetation, which hinders in-stream primary production, but supplies large inputs of leaf litter that constitute the main source of energy, nutrients and carbon for the aquatic biota (Vannote et al. 1980; Wallace et al. 1997; Abelho 2001). These particulate inputs are incorporated into aquatic food webs through litter decomposition, a key ecosystem process where microbial decomposers (mainly aquatic hyphomycetes) and invertebrate shredders play a key role (Hieber and Gessner 2002). Microbial decomposers in particular are central to litter decomposition as they promote litter mass loss directly by mineralizing organic carbon, converting it into biomass and promoting the release of fine particulate organic matter (Gulis and Suberkropp 2003; Cornut et al. 2010). Further, microbial activities on the litter (biomass build-up, immobilization of dissolved nutrients, and enzymatic maceration) increase litter palatability to shredders thus facilitating the incorporation of litter carbon into secondary production (Canhoto and Graça 2008; Bärlocher and Sridhar 2014).

In native forests, litter inputs to streams are generally diverse (Swan and Palmer 2004; Lecerf et al. 2005; Molinero and Pozo 2006; Ferreira et al. 2016). Distinct litter species differ in their physical and chemical characteristics,

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with soft litter that has low carbon-to-nutrients ratios and low lignin concentration generally being colonized and decomposed faster than more recalcitrant litter (Gessner and Chauvet 1994; Schindler and Gessner 2009; Ferreira et al. 2012; Frainer et al. 2015). Since microbes differ in their enzymatic capabilities (Arsuffi and Suberkropp 1984, 1988; Chandrashekar and Kaveriappa 1988), nutrient requirements (Bisht 2013) and morphology (Dang et al. 2007), different litter species can support distinct microbial communities (Canhoto and Graça 1995; Gulis 2001; Ferreira and Graça 2016). Diverse litter mixtures can thus support higher diversity of decomposers as substrate heterogeneity allows for niche complementarity (Laitung and Chauvet 2005; Lecerf et al. 2005; Kominoski et al. 2009; Ferreira et al. 2016). Effects of litter mixing on litter decomposition are, however, difficult to anticipate and may depend on species identity (Lecerf et al. 2011). Some studies have reported additive effects of species mixing on litter decomposition (i.e. there is no interaction between component litter species in the mixture; Schindler and Gessner 2009; Bruder et al. 2011; Ferreira et al. 2012; Frainer et al. 2015; Ferreira and Graça 2016). Other studies have found non-additive effects of litter mixing with litter decomposition in mixtures being accelerated or decelerated when compared with expectations based on the decomposition of the component litter species incubated individually (Kominoski et al. 2010; Lecerf and Richardson 2010; Lecerf et al. 2011). Non-additive effects are likely to arise when the leaf litter species within a mixture contrast in their functional traits (Gessner et al. 2010; Handa et al. 2014; but see, e.g. Frainer et al. 2015), and the potential mechanisms for this include: (a) leaching of inhibitory or stimulatory compounds that can influence microbial colonization and activity in the neighbour litter species, (b) nutrient transfer between different litter species by fungi, (c) complementary resource use by shredders, which also benefit from a more diverse habitat, and (d) protection of soft litter from physical fragmentation by tough litter species (Kominoski et al. 2009; Gessner et al. 2010; Lecerf et al. 2011; Chapman et al. 2013; Ferreira and Graça 2016). Several of these mechanisms may occur simultaneously, and eventually result in apparent additive effects of litter mixing on the decomposition of the litter mixture if the decomposition on component litter species is affected in opposite directions (e.g. the decomposition of one species is stimulated while the decomposition of the neighbour species is proportionally inhibited in a two-species litter mixture). To properly identify additive effects of litter mixing, the decomposition of component litter species in mixtures needs to be assessed and compared with the decomposition of the litter species decomposing individually (Bruder et al. 2011; Ferreira et al. 2012; Frainer et al. 2015; Ferreira and Graça 2016).

In addition, litter-mixing effects on litter decomposition may depend on the environmental conditions, which could partially explain the contrasting results reported among studies (McArthur et al. 1994; Leroy and Marks 2006; Lecerf et al. 2011; Ferreira and Graça 2016). Current velocity, in particular, presents a great spatial and temporal heterogeneity, even within a single stream, and it is considered an important factor affecting leaf litter decomposition, with effects being stronger at latter stages of the decomposition process when leaf litter is soft and more susceptible to physical abrasion (Ferreira et al. 2006; Bastias et al. 2020). Current velocity is also recognized as an important factor regulating fungal reproduction and community structure associated with leaf litter (Ferreira and Graça 2006). Therefore, mixing leaf litter of contrasting characteristics (e.g. soft and tough) may induce stronger litter-mixing effects on fast than on slow current velocity conditions, as tough litter could protect soft litter from physical abrasion at fast current velocity conditions (Abelho 2009). Mixing effects may be stronger at advanced decomposition stages, when litter is more fragile to physical abrasion (Bastias et al. 2020).

In this study, we assessed litter-mixing effects on litter decomposition and associated microbial activity and aquatic hyphomycete community structure by considering two contrasting leaf litter species incubated individually and in a mixture under two different current velocity conditions in a forest stream. Component litter species from the mixture were processed individually, which allowed testing the following hypotheses: (a) mixing of contrasting litter species induces non-additive effects on litter decomposition and associated decomposers (activity and community structure), i.e. values differ when litter is incubated in the mixture or individually, (b) mixing effects are stronger under fast current velocity conditions.

Methods

Study stream

The study was carried out in a second-order forest stream located in Central Portugal (Ribeira da Sardeira, Lousã Mountain; 40° 5′ 21″ N, 8° 12′ 6″ W, 520 m asl). The stream drains an area of 2.9 km^2 underlined by schist bedrock and covered by a mixed deciduous forest dominated by chestnut (*Castanea sativa* Mill.) and oak (*Quercus robur* L.), and where human activity is low. This stream has long been used as a reference stream by the research team, and has circumneutral pH, low conductivity, and low nutrient concentrations (Gulis et al. 2006; Ferreira et al. 2016). Substrate is mostly composed of pebbles and cobles.

For this study, five locations with slow current velocity and five locations with fast current velocity were selected

along a ~ 200-m stream reach using a current metre (Valeport 15277, Valeport Lda., UK), so that current velocity would differ by one order of magnitude between slow and fast treatments, but depth would be similar. During the study, current velocity at these locations was monitored on five occasions. On the same occasions, oxygen saturation, temperature, electrical conductivity and pH of stream water were determined in situ using a multi-parametric sensor (WTW, Weilheim, Germany).

Leaf litter

Alder (A; *Alnus glutinosa* (L.) Gaertner) and oak (O; *Quercus robur* L.) leaves were collected after senescence in autumn 2007 near Coimbra, Central Portugal. Leaf litter was air-dried at room temperature in the dark and stored in paper boxes until used. These tree species were chosen because they are common in riparian forests in Central Portugal and throughout Europe (Graça and Poquet 2014), and have contrasting litter characteristics that result in distinct litter decomposition rates (faster for alder than oak; e.g.Ferreira et al. 2012; Woodward et al. 2012). The contrasting litter characteristics, concerning both physical aspects (alder leaves are softer than oak leaves) and chemical composition (alder leaves are richer in nitrogen while oak leaves are richer in lignin and polyphenolics) (Graça and Poquet 2014), may favour interactions between litter species when incubated together.

Initial leaf litter toughness was determined from five individual leaves from each species, after leaves had been soaked in distilled water for 1 h, using a penetrometer and results were expressed as the mass (g) needed to perforate the leaf with an iron rod (Bärlocher et al. 2020). Specific leaf area (SLA) was determined from 12-mm diameter litter discs, cut from moistened leaves with a cork borer, as the disc area (mm^2) to dry mass (mg) ratio. Initial concentrations (% dry mass) of lignin (Goering and Van Soest 1970), total polyphenols and phosphorus (Bärlocher et al. 2020), and nitrogen and carbon (CN auto-analyser; IRMS Thermo Delta V advantage with a Flash EA-1112 series; Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to characterize the initial chemical quality of the litter.

Leaf litter decomposition

Air-dried leaves from the two selected species were enclosed in coarse mesh bags (15 × 20 cm, 10-mm mesh opening), individually and in mixtures (three litter treatments in total: A, O, AO). Litter bags were prepared with 3.00 g (± 0.05) of leaves; in mixtures, litter mass was divided equally by the component litter species (i.e. 1.50 g each). In early May 2015, 4 litter bags per litter treatment were nailed to the stream bed in each of 5 locations with slow and 5 locations with fast current velocity (120 bags total, 4 bags × 3 litter treatments × 2 current velocities × 5 locations). Five replicate litter bags (one from each location) per litter treatment and current velocity condition were retrieved from the stream on four occasions, after 11, 18, 26 and 32 days of incubation. Litter bags were enclosed individually in plastic zip lock bags, stored on ice and returned to the laboratory where they were promptly processed.

In the laboratory, component litter species from litter mixtures were processed individually (Ostrofsky 2007). Litter was gently rinsed with distilled water on top of a 500-µm mesh sieve to retain small litter fragments, and fifteen discs (12-mm diameter) were cut out with a cork borer to assess microbial respiration rates, fungal biomass, and aquatic hyphomycete sporulation rates and species richness (see below), except on day 32 when no litter discs were taken due to the small amount of alder litter remaining. Remaining litter was oven-dried at 105 °C for 24 h and weighed (± 0.1 mg) to determine litter dry mass (DM). Subsequently, litter was ignited at 500 °C for 4 h and ashes were weighed (± 0.1 mg). Litter ash-free dry mass (AFDM) was determined as the difference between DM and ash mass. The fraction of AFDM remaining, considering the discs removed for microbial determinations (see below), was estimated as AFDM at the sampling date/initial AFDM. Initial AFDM was estimated from initial air-dry mass by applying a conversion factor. The air-dry mass to AFDM conversion factor was obtained from an extra set of three replicates of each litter treatment that was incubated in the stream for 10 min on day 0. These bags were returned to the laboratory and AFDM was determined as described above.

Microbial respiration

Microbial respiration rates were determined for each litter species incubated individually and in mixtures, and used as a measure of overall microbial activity. Microbial respiration rates were determined promptly using a closed six-channel dissolved oxygen (O_2) measuring system (Strathkelvin 929 System, North Lanarkshire, UK) connected to a computer (Bärlocher et al. 2020). The O_2 electrodes were calibrated against a saturated solution of sodium sulphite in 0.01 M sodium borate (0% O_2) and 100% O_2-saturated stream water maintained at 15 °C. Five litter discs were incubated in 3-mL chambers filled with 100% O_2-saturated stream water, homogenized with a magnetic stirring bar, and kept at 15 °C by circulation of water originating from a temperature-controlled water bath. Additional chambers without litter discs were used as controls. Respiration rates were determined by the difference in O_2 concentration in the sample and the control chambers over a 20-min interval during which O_2 consumption over time was linear, corrected for the chamber volume, time, and litter discs mass. The litter discs were

oven-dried at 105 °C for 24 h, weighed (± 0.1 mg), ignited (4 h at 500 °C) and reweighed (± 0.1 mg) for determination of discs AFDM. Results were expressed as mg O_2 g^{-1} litter AFDM h^{-1}.

Fungal biomass

Five litter discs from each litter species incubated individually and in mixtures were frozen at – 20 °C until ergosterol extraction, whose concentration can be used as a surrogate for fungal biomass (Gessner and Chauvet 1993). Discs were freeze-dried and weighed (± 0.1 mg) just before ergosterol extraction for determination of discs DM; discs DM was converted into discs AFDM using the ash fraction of discs used to induce conidial production (see below). Since current velocity had no effect on litter decomposition and microbial respiration rates (see "Results") only samples from fast current velocity conditions were processed further. Litter discs were immersed in alkaline methanol (8 g KOH L^{-1}) and heated in a water bath (80 °C, 30 min). The extracted lipids were purified by solid-phase extraction (Waters Sep-Pak® Vac RC tC_{18} cartridges; Waters Corp., Milford, Massachusetts, USA) and quantified by high-performance liquid chromatography (HPLC) by measuring absorbance at 282 nm (Bärlocher et al. 2020). The HPLC system (Dionex, Sunnyvale, California, USA) was equipped with the Thermo Scientific Syncronis C_{18} column (250 × 4 mm, 5-µm particle size) and the Thermo Universal Unigard holder 4/4.6 mm ID3 + Syncronis C_{18} (10 × 4 mm, 5-µm particle size) drop in guard pre-column (Thermo, Waltham, Massachusetts, USA). The working temperature was 33 °C and the sample was carried out by 100% methanol, flowing at 1.4 mL min^{-1}. Ergosterol was converted into fungal biomass assuming 5.5 µg ergosterol mg^{-1} fungal dry mass (Gessner and Chauvet 1993), and results were expressed as mg fungal biomass g^{-1} litter AFDM.

Conidial production by aquatic hyphomycetes

Conidial production by aquatic hyphomycetes was induced for each litter species incubated individually and in mixtures, and use as an indicator of fungal reproductive activity. Five litter discs were placed in 100-mL Erlenmeyer flasks with 25 mL of filtered (glass fibre filters, 47 mm diameter, pore size 0.7 µm; Whatman GF/F, GE Healthcare UK Limited, Little Chalfont, UK) stream water. The flasks were incubated for 48 h on a shaker (100 rpm) at 15 °C and with 12-h light:12-h dark photoperiod. The conidial suspensions were then poured into 50-mL Falcon tubes, the flasks rinsed twice with distilled water, the suspensions fixed with 2 mL of 37% formalin, the sample volume adjusted with distilled water to 35 mL, and the tubes stored in the dark until conidial identification (Bärlocher et al. 2020). The litter discs were oven-dried at 105 °C for 24 h, weighed (± 0.1 mg), ignited (4 h at 500 °C) and reweighed (± 0.1 mg) for determination of discs AFDM.

Current velocity had no effect on litter decomposition and microbial respiration rates (see "Results") and so only conidial samples from fast current velocity conditions were processed further. To prepare the slides for counting and identifying the conidia, 100 µL of Triton X–100 (0.5%) were added to the suspensions and mixed at ~ 150 rpm for ~ 2 min with a magnetic stirring bar. An aliquot of the suspensions was then filtered (SMWP membrane filters, 5-µm pore size; Millipore Corp., Billerica, MA, USA) with gentle vacuum and the filters were stained with 0.05% cotton blue in 60% lactic acid. Slides were scanned with a microscope (SM-Lux, Leitz, Wetzlar, Germany) at 200 × magnification (Bärlocher et al. 2020) for conidia identification and counting. Sporulation rates were expressed as number of conidia released mg^{-1} litter AFDM day^{-1}, and aquatic hyphomycete species richness was expressed as number of species $sample^{-1}$.

Data analysis

Initial litter characteristics were compared between litter species with two-tailed Student's *t* tests. Current velocity ($\log(x+1)$-transformed) and depth over time were compared between slow and fast current velocity conditions with two-way analysis of variance (ANOVA), with time and current velocity condition as the categorical variables.

Litter decomposition rates (k) were estimated by negative linear regressions between the fraction of AFDM remaining (ln-transformed) and time (days), for each litter treatment (A, O, AO) and for each component species in the litter mixture (A (AO), O (AO)). Comparisons of decomposition rates of litter species (a) when incubated individually (A vs O), (b) when incubated in the mixture (A (AO) vs O (AO)), or (c) when incubated individually or in the mixture (A vs A (AO) and O vs O (AO)), under slow and fast current velocity conditions, were done by two-way analysis of covariance (ANCOVA), using the fraction of AFDM remaining (ln-transformed) as the dependent variable, time as the covariate, and litter treatment and current velocity condition as the categorical variables. When needed, subsequent pairwise comparisons were performed using Tukey's Honest Significant Difference (HSD) test. The same comparisons were applied to microbial respiration rates using a three-way analysis of variance (ANOVA), with time, litter treatment and current velocity as the categorical variables.

Fungal biomass ($\log(x+1)$-transformed), aquatic hyphomycetes sporulation rates ($\log(x)$-transformed) and species richness associated with decomposing leaf litter incubated at the fast current velocity condition were compared among litter treatments with two-way ANOVA, with time and litter treatment as the categorical variables, to test if the microbial

variables differed (a) between litter species incubated individually (A vs O), (b) between litter species incubated in the mixture (A (AO) vs O (AO)), or (c) between litter species incubated individually or in the mixture (A vs A (AO) and O vs O (AO)). Aquatic hyphomycete community structure was compared (a) between litter species incubated individually (A vs O), (b) between litter species incubated in the mixture (A (AO) vs O (AO)), or (c) between litter species incubated individually or in the mixture (A vs A (AO) and O vs O (AO)) by analysis of similarity (ANOSIM; PAST software package, PAlaeontological STatistics, ver. 3).

Before statistical analyses, the distributional properties of the data were assessed to identify outliers. The Shapiro–Wilk's test was applied to assess normality and the Bartlett's test to assess homogeneity of variances, and data were transformed when needed. All statistical analyses were conducted using R version 3.5.2 (R Core Team, 2018), except when indicated otherwise, with a significance level set at $\alpha = 0.05$ for all tests.

Results

Study stream

The study stream had relatively cool water (12.5 ± 0.9 °C, mean ± SE), low conductivity (39.2 ± 1.5 µS cm^{-1}), and oxygen saturation around 75%. The mean depth during the experiment was 10.8 ± 0.5 cm (mean ± SE) across sampling locations and did not significantly differ between slow and fast current velocity conditions over time (two-way ANOVA, $F = 0.15$, $df = 4$, $p = 0.960$). The mean current velocity across sampling dates was 0.03 ± 0.01 m s^{-1} (mean ± SE) in slow current locations and 0.84 ± 0.08 m s^{-1} in fast current locations, and significantly differed between locations (two-way ANOVA, $F = 4.27$, $df = 4$, $p = 0.005$). Current velocity decreased over the incubation period (two-way ANOVA, $F = 11.14$, $df = 4$, $p < 0.001$), but it was always two orders of magnitude faster in the fast than in the slow current locations. Specifically, it decreased over the litter incubation period from 1.1 ± 0.06 m s^{-1} on day 0 to 0.7 ± 0.05 m s^{-1} (mean ± SE) on day 32 in fast current locations, and from 0.06 ± 0.005 m s^{-1} on day 0 to 0.006 ± 0.006 m s^{-1} on day 32 in slow current locations.

Leaf litter

Alder leaf litter had significantly higher SLA and nitrogen concentration than oak (Table 1). Contrarily, oak was tougher and had higher lignin, polyphenolics and phosphorus concentration than alder (Table 1). No significant difference was found for carbon concentration.

Table 1 Initial characterization (mean ± SE, $n = 3$) of the leaf litter species used in the litter decomposition experiment

Litter variables	Alder (A)	Oak (O)	*p*
Toughness (g)	107.2 ± 8.6	157.2 ± 11.3	0.008
SLA (mm^2 mg^{-1} DM)	19.3 ± 0.6	15.9 ± 1.0	**0.022**
Lignin (% DM)	35.8 ± 0.4	39.7 ± 0.6	**0.005**
Polyphenols (% DM)	3.5 ± 03	16.4 ± 0.6	**< 0.001**
Phosphorus (% DM)	0.1 ± 0.0	0.6 ± 0.0	**< 0.001**
Nitrogen (% DM)	2.5 ± 0.1	0.9 ± 0.1	**< 0.001**
Carbon (% DM)	47.5 ± 1.0	48.0 ± 0.2	0.613
C:N (molar ratio)	22.4 ± 0.4	59.9 ± 3.7	**< 0.001**
C:P (molar ratio)	875.8 ± 17.8	210.2 ± 0.8	**< 0.001**

Comparisons between leaf litter species were made with two-tailed t tests and p values are shown

SLA specific leaf area, *DM* dry mass

Statistical significances are highlighted in bold

Leaf litter decomposition

Litter mass remaining decreased exponentially over time and, after 32 days incubation, it varied between 13 ± 3 and 18 ± 4% (mean ± SE) for alder, between 57 ± 3 and 62 ± 3% for oak, and between 29 ± 4 and 39 ± 3% for the mixture (Fig. 1), which translated into decomposition rates between 0.0104 and 0.0289 day^{-1} across litter treatments and current velocity conditions (Table 2). Decomposition rates significantly differed between litter species, with alder litter decomposing faster than oak litter when incubated individually (A > O) and when incubated in the mixture (A (AO) > O (AO)), under both slow and fast current velocity conditions (Fig. 1, Tables 2 and 3). Decomposition rates of individual litter species were not significantly affected by litter mixing (A–A (AO) and O–O (AO)) under both current velocity conditions (Fig. 1, Tables 2 and 3), although oak litter in mixture had lower mass remaining than oak incubated individually (O (AO) < O) on the last sampling date under fast current velocity conditions (Fig. 1, Table 3). Decomposition rates of alder litter were not significantly affected by current velocity (Fig. 1, Tables 2 and 3), while those of oak (O and O (AO)) were significantly higher in fast than in slow current velocity conditions (Fig. 1, Tables 2 and 3).

Microbial activity

Microbial respiration rates were not significantly affected by current velocity in any of the comparisons assessed (Fig. 2, Table 4). Microbial respiration rates were significantly affected by the interaction between time and litter species when litter species were incubated individually, with higher values on alder than on oak (A > O) on day 11, while only litter species affected respiration rates when litter was

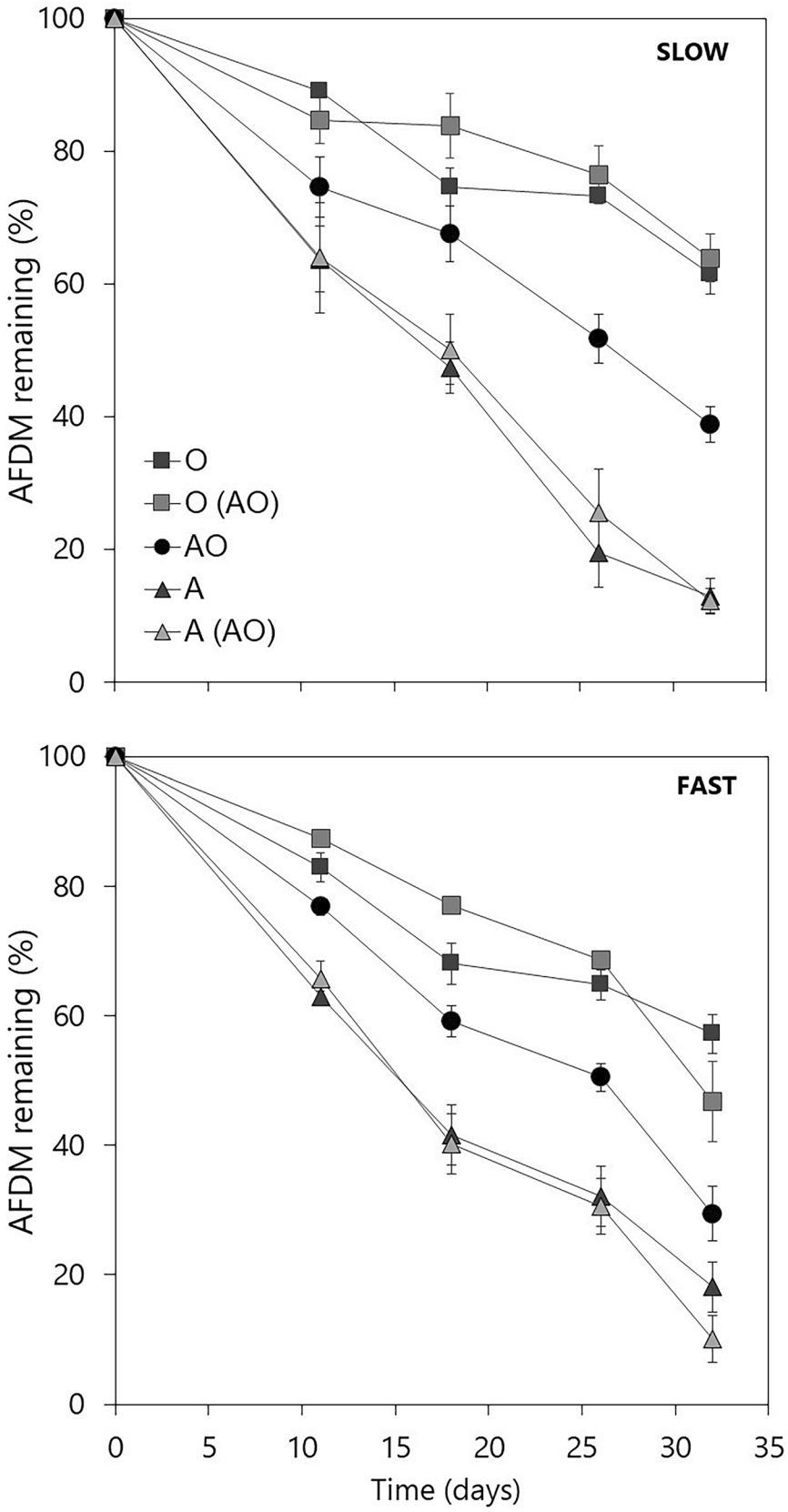

Fig. 1 Percentage of ash-free dry mass (AFDM) remaining (mean ± SE, $n = 5$) over time for alder and oak leaf litter incubated individually (A and O, respectively) and in mixture (A (AO) and O (AO), respectively), and for the litter mixture (AO), under slow and fast current velocity conditions

Table 2 Decomposition rates (k) for alder and oak leaf litter incubated individually (A and O, respectively) and in mixtures (A (AO) and O (AO), respectively), and for the litter mixture (AO), under slow and fast current velocity conditions

Litter treatment	Slow			Fast		
	k (day^{-1})	SE	R^2	k (day^{-1})	SE	R^2
Single						
A	0.0289	0.0009	0.81	0.0273	0.0010	0.66
O	0.0117	0.0005	0.76	0.0142	0.0006	0.63
Mixture						
AO	0.0190	0.0008	0.73	0.0209	0.0006	0.87
A (AO)	0.0282	0.0012	0.72	0.0285	0.0009	0.78
O (AO)	0.0104	0.0008	0.43	0.0139	0.0007	0.77

Standard error (SE) and coefficient determination (R^2) of the regressions are also shown; $p < 0.001$ in all cases

incubated in the mixture with higher values on alder than on oak (A (AO) > O (AO)) (Fig. 2, Table 4). Litter mixing, however, did not have an effect on alder (A–A (AO)) or oak (O–O (AO)) microbial respiration rates (Fig. 2, Table 4).

Fungal biomass significantly differed between litter species when incubated individually or in the mixture depending on sampling date (significant time × litter treatment interaction; Fig. 3A, Table 5), with A > O on day 11 and A < O on day 26. Litter mixing did not have an effect on alder (A–A (AO)) or oak (O–O (AO)) fungal biomass (Fig. 3A, Table 5).

Sporulation rates by aquatic hyphomycetes were significantly affected by litter species when incubated individually or in the mixture, but effects depended also on sampling date (significant time × litter treatment interaction; Table 5), with A > O on day 11 and A < O on days 18 and 26 (Fig. 3B). Litter mixing did not have an effect on alder (A–A (AO)) or oak (O–O (AO)) sporulation rates (Fig. 3B, Table 5).

Aquatic hyphomycete community

Mean species richness of released aquatic hyphomycete conidia was significantly higher on alder litter than on oak litter when incubated individually (A > O) (Table 6), but not when incubated in the mixture (A (AO)–O (AO)) (Table 4). Litter mixing, did not have an effect on alder (A–A (AO)) or oak (O–O (AO)) aquatic hyphomycete species richness (Table 6). Cumulative species richness was 13 for both species at the end of the incubation period (Fig. 4).

Flagellospora curvula Ingold (42% of total conidial abundance), followed by *Stenocladiella neglecta* Marvanová & Descals (19–21%) and *Tetrachaetum elegans* Ingold (12–15%) contributed the most to conidial production associated with alder leaf litter, incubated individually or in the mixture (Fig. 4). In contrast, the species that most contributed to conidial production associated with oak leaf litter, incubated individually or in mixture, was *T. elegans* (41–45%), followed by *Hydrocina chaetocladia* Scheuer (21–24%) and *Lunulospora curvula* Ingold (11–14%), and to a lesser extent by *F. curvula* (8–9%) (Fig. 4). Aquatic hyphomycete community structure significantly differed between alder and oak litter when incubated individually or in the mixture (A ≠ O and A (AO) ≠ O (AO)) (Fig. 4, Table 6). Community structure for each litter species did not significantly differ when litter was incubated individually or in the mixture (A–A (AO) and O–O (AO)) (Fig. 4, Table 6).

Discussion

Mixing of contrasting litter species has been reported to induce non-additive effects on leaf litter decomposition (Gessner et al. 2010), especially due to differences in the performance of the microbial decomposer community (Chapman et al. 2013) and detritivores (Vos et al. 2011). However, the actual influence of leaf litter diversity on litter decomposition in streams remains unclear, and the mechanisms behind the diversity effects and the influence that environmental factors could exert on them have been poorly examined (Swan and Palmer 2004; Leroy and Marks 2006; Schindler and Gessner 2009; Lecerf et al. 2011; Frainer et al. 2015). In this study, we assessed litter-mixing effects on litter decomposition and associated microbial activity and aquatic hyphomycete community structure of two contrasting leaf litter species (alder and oak), under two different current velocity conditions, an important environmental driving factor of leaf litter decomposition in streams (Ferreira and Graça 2006; Bastias et al. 2020).

In our study, decomposition rates of individual litter species were not generally affected by litter mixing under both current velocity conditions. Therefore, our results provided weak evidences for diversity effects on leaf litter decomposition in streams, in accordance with previous studies that also reported additive effects on litter decomposition when mixing contrasting leaf litter species (Schindler and Gessner 2009; Bruder et al. 2011; Frainer et al. 2015; Santschi et al. 2018; but see Abelho 2009; Sanpera-Calbet et al. 2009; Ferreira et al. 2012). The lack of litter-mixing effects on litter decomposition in the present study occurred even though the

Table 3 Summary of two-way ANCOVAs (litter treatment and current velocity as categorical variables, time as the continuous variable) on fraction of AFDM remaining (ln-transformed) to test whether decomposition rates differed between litter species (a) when incubated individually (A *vs* O), (b) when incubated in the mixture (A (AO) vs O (AO)), or (c) when incubated individually or in the mixture (A vs A (AO) and O vs O (AO)), under slow and fast current velocity conditions

Source of variation	*df*	*F*	*p*
A vs O			
Time	1	90.08	**< 0.001**
Treatment	1	131.74	**< 0.001**
Velocity	1	0.94	0.336
Time × treatment	1	38.53	**< 0.001**
Time × velocity	1	2.27	0.137
Treatment × velocity	1	4.68	**0.030**
Time × treatment × velocity	1	2.62	0.109
Residuals	72		
A (AO) vs O (AO)			
Time	1	126.69	**< 0.001**
Treatment	1	175.50	**< 0.001**
Velocity	1	0.01	0.951
Time × treatment	1	46.21	**< 0.001**
Time × velocity	1	0.01	0.947
Treatment × velocity	1	2.19	0.143
Time × treatment × velocity	1	3.03	0.090
Residuals	71		
A vs A (AO)			
Time	1	152.32	**< 0.001**
Treatment	1	0.02	0.900
Velocity	1	0.47	0.104
Time × treatment	1	0.01	0.920
Time × velocity	1	0.59	0.070
Treatment × velocity	1	0.50	0.483
Time × treatment × velocity	1	0.39	0.536
Residuals	71		
O vs O (AO)			
Time	1	119.17	**< 0.001**
Treatment	1	1.06	0.306
Velocity	1	14.09	**< 0.005**
Time × treatment	1	1.29	0.260
Time × velocity	1	4.84	**0.031**
Treatment × velocity	1	0.08	0.772
Time × treatment × velocity	1	0.06	**0.042**
Residuals	72		

Bold *p* values indicate significant effects

leaf litter species used showed contrasting functional traits and differed greatly in decomposition rates when incubated individually, which are considered prerequisites for diversity effects on decomposition to arise (Schindler and Gessner 2009; Lecerf et al. 2011). Alder leaf litter was softer, and had significantly higher nitrogen concentration and lower lignin concentration than oak. Being a less challenging substrate, alder leaf litter was colonized and decomposed faster than oak when incubated individually, as often reported (Gessner and Chauvet 1994; Gulis et al. 2006; Ferreira et al. 2012; Woodward et al. 2012; Pereira et al. 2016). The same colonization and decomposition pattern (i.e. faster colonization and decomposition for alder than for oak) was observed on the litter mixture, suggesting that litter decomposition was not affected by mixing contrasting leaf litter species. The lack of mixing effects on litter decomposition was further supported by the lack of differences in colonization and decomposition rates between each species incubated individually and in the mixture. Mixing contrasting leaf litter species had no significant effect for any of the microbial parameters assessed, suggesting that microbial communities on litter species did not interact or mutually influence each other in the litter mixture. Therefore, despite mixing leaf litter species widely differing in litter quality, the proposed microbial-mediated mechanisms of diversity effects on decomposition (i.e. microbial colonization influenced by leaching of inhibitory or stimulatory compounds from neighbour litter species, or nutrient transfer between different litter species by fungi; Kominoski et al. 2009; Gessner et al. 2010) did not arise in our study. Our results are in line with those of Frainer et al. (2015), who found that fungal biomass was largely dependent on the litter species in which it was growing, rather than on the trait dissimilarity of the litter mixture (but see Kominoski et al. (2009)). Frainer et al. (2015) suggested that a lack of diversity effects on fungal decomposers could be also explained by an allocation of resources to fungal reproduction instead of biomass, but our results indicated that sporulation rates were not affected by litter mixture neither, as previously observed (Bruder et al. 2011; Ferreira et al. 2016; but see Fernandes et al. 2012).

Despite the general lack of litter-mixing effects on leaf litter decomposition rates, litter-mixing effects on percentage litter mass remaining were detected at the end of the incubation period, when oak litter in the mixture had lower percentage mass remaining than oak incubated individually under fast current velocity conditions. These results suggested that current velocity and incubation time might influence diversity effects on leaf litter decomposition in streams. Current velocity can enhance leaf litter decomposition by stimulating fungal activity (Ferreira and Graça 2006) and promoting physical abrasion (Ferreira et al. 2012; Fonseca et al. 2013), specially at late stages of the decomposition process (Bastias et al. 2020) when leaves are more fragile due to microbial-mediated enzymatic maceration. By

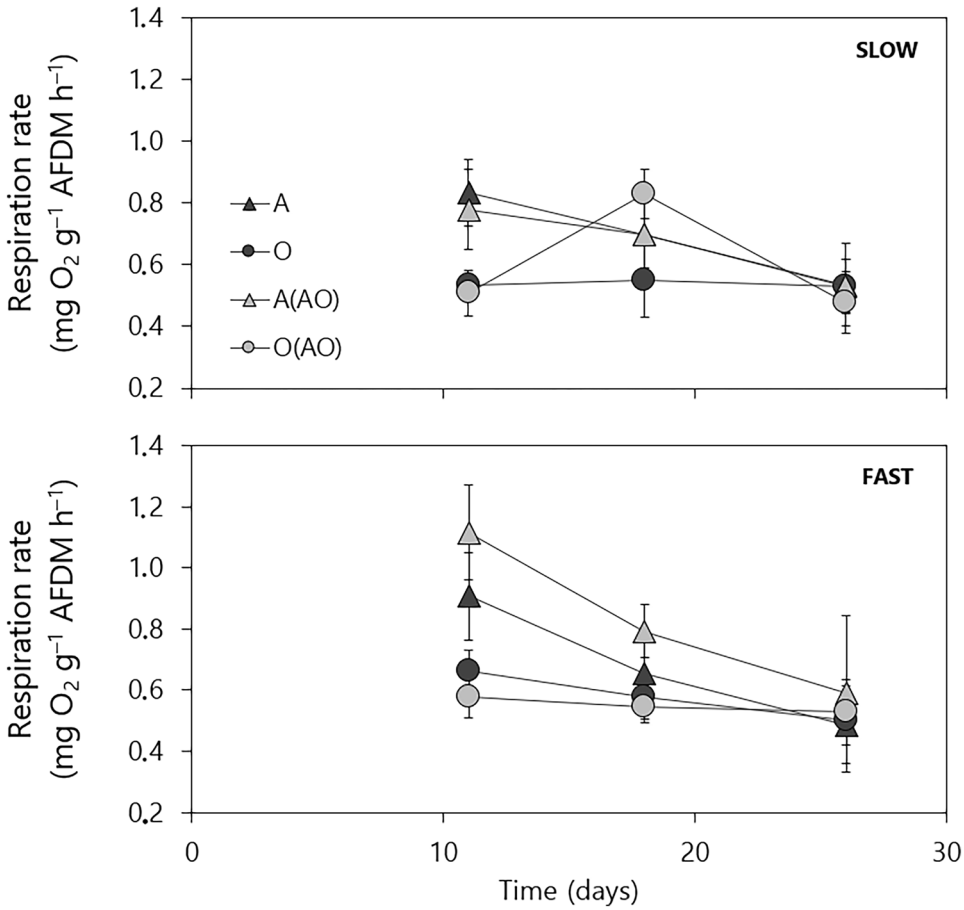

Fig. 2 Microbial respiration rates (mean ± SE, $n = 5$) associated with alder and oak litter incubated individually (A and O, respectively) or in the mixture (A (AO) and O (AO), respectively) under slow and fast current velocity conditions over time

contrast, slower flow velocities may decrease fungal activity and development via reduced fluxes of dissolved oxygen and nutrients (Bruder et al. 2016) and increased fine sediment deposition (Martínez et al. 2020), that can also limit macroinvertebrate activity (Rabeni et al. 2005), compromising the overall decomposition activity. All these mechanisms could explain the flow velocity effects observed on oak litter decomposition under fast current velocity conditions. However, due to its higher softness, we expected that alder leaf litter would be more susceptible to flow-related abrasion than oak leaf litter, and that litter-mixing effects would arise specially under fast current velocity conditions, as oak litter (tough) would protect alder litter (soft) from physical abrasion. Contrary to our predictions, decomposition rates of alder leaf litter were not affected by current velocity, while those of oak were significantly higher in fast than in slow current velocity conditions. One plausible explanation behind these results could be related to the fast decomposition rates observed for alder leaf litter (~ 13% AFDM remaining after 32 days of incubation), which might have masked potential effects of current velocity and litter mixing on its decomposition. Our study was carried out in late spring-early summer, and the gradual increase in water temperature naturally occurring in this season likely stimulated the microbial activity and the overall decomposition process (Ferreira et al. 2014). In addition, in late spring-early summer, benthic organic matter is reduced in temperate streams (Pozo et al. 1997) and litter bags used in our experiment might have attracted detritivores, further enhancing leaf litter decomposition. As Swan and Palmer (2004) pointed out, seasonality might have influenced the direction and magnitude of diversity effects on leaf litter decomposition in our study. At the same time, the diversity effects observed in oak litter at late stages of the decomposition process could be also related to differences in the leaf pack size between treatments (i.e. leaf litter incubated individually or in mixtures). Past studies have demonstrated that decomposition rates decrease as leaf pack size increases mainly due to limited physical abrasion, oxygen diffusion and shredders accessibility to leaf tissue in the middle of large leaf packs (Richardson and Chauvet 2019). In our study, most of the alder litter was completely decomposed

Table 4 Summary table for three-way ANOVAs (time, litter treatment and current velocity as categorical variables) on microbial respiration rates associated with leaf litter decomposing under fast current velocity conditions, to test whether microbial respiration differed between litter species (a) when incubated individually (A vs O), (b) when incubated in the mixture (A (AO) vs O (AO)), or (c) when incubated individually or in the mixture (A vs A (AO) and O vs O (AO))

Source of variation	*df*	*F*	*p*
A vs O			
Time	1	10.07	**0.002**
Treatment	1	5.05	**0.029**
Velocity	1	0.12	0.730
Time × treatment	1	4.36	**0.041**
Time × velocity	1	0.92	0.341
Treatment × velocity	1	0.21	0.643
Time × treatment × velocity	1	0.01	0.930
Residuals	50		
A (AO) vs O (AO)			
Time	1	7.59	**0.008**
Treatment	1	8.45	**0.005**
Velocity	1	0.47	0.493
Time × treatment	1	3.38	0.072
Time × velocity	1	1.07	0.305
Treatment × velocity	1	2.46	0.123
Time × treatment × velocity	1	0.38	0.538
Residuals	47		
A vs A (AO)			
Time	1	16.02	**< 0.001**
Treatment	1	0.93	0.338
Velocity	1	1.07	0.304
Time × treatment	1	0.04	0.841
Time × velocity	1	1.09	0.301
Treatment × velocity	1	1.22	0.274
Time × treatment × velocity	1	0.18	0.675
Residuals	48		
O vs O (AO)			
Time	1	1.74	0.192
Treatment	1	0.01	0.951
Velocity	1	0.02	0.882
Time × treatment	1	0.00	0.962
Time × velocity	1	0.82	0.368
Treatment × velocity	1	1.13	0.293
Time × treatment × velocity	1	0.10	0.746
Residuals	51		

Statistical significances are highlighted in bold

at the end of the experiment, and therefore, the size of the leaf packs containing oak litter in mixtures were about half the size of monospecific oak leaf litter packs.

Previous studies indicated that incubation time is also a key factor for capturing diversity effects on leaf litter decomposition (Lecerf et al. 2011; Fernandes et al. 2013). This could be explained by the fact that detritivores are more likely to generate strong non-additive effects on leaf litter decomposition than early microbial colonizers such as fungi (Sanpera-Calbet et al. 2009; Lecerf et al. 2011), specially at late stages of the decomposition process when detritivores drive leaf litter degradation (Gessner et al. 1999). In this regard, Sanpera-Calbet et al. (2009) observed that the presence of a refractory litter in mixtures lead to higher shredder abundance than expected, suggesting that litter mixtures could promote diversity effects due to alterations in shredder-mediated decomposition. In our study, the presence of oak as refractory litter might have triggered increased detritivores abundance in mixture litter bags, which could have enhanced oak litter decomposition at the end of the incubation period in the mixture, when alder litter was almost completely decomposed and oak litter was more palatable due to the microbial conditioning and the physical abrasion promoted under fast current velocity conditions. In addition, previous studies pointed out that, along a stream reach, fast current velocity conditions tend to hold higher shredder abundance than slow current velocity conditions (Graça et al. 2004; Ferreira et al. 2006). Therefore, at the end of the incubation period, when alder leaf litter was almost completely decomposed, the combined effects of higher shredder activity and physical abrasion might have promoted oak litter mass loss in mixture litter bags under fast current velocity conditions, leading to faster decomposition of oak litter incubated in the mixture than individually.

To conclude, our study reported weak effects of litter mixture on leaf litter decomposition, despite contrasting functional traits between the leaf litter species used. Mixing contrasting leaf litter species had no significant effect on microbial decomposers, indicating that decomposition rates and microbial colonization and activity depended primarily on the traits of the target litter species and were not affected by those of the companion species. However, litter-mixing effects on litter mass remaining were detected on oak litter at late decomposition stages under fast current velocity conditions, suggesting that both current velocity and the incubation time can influence diversity effects on

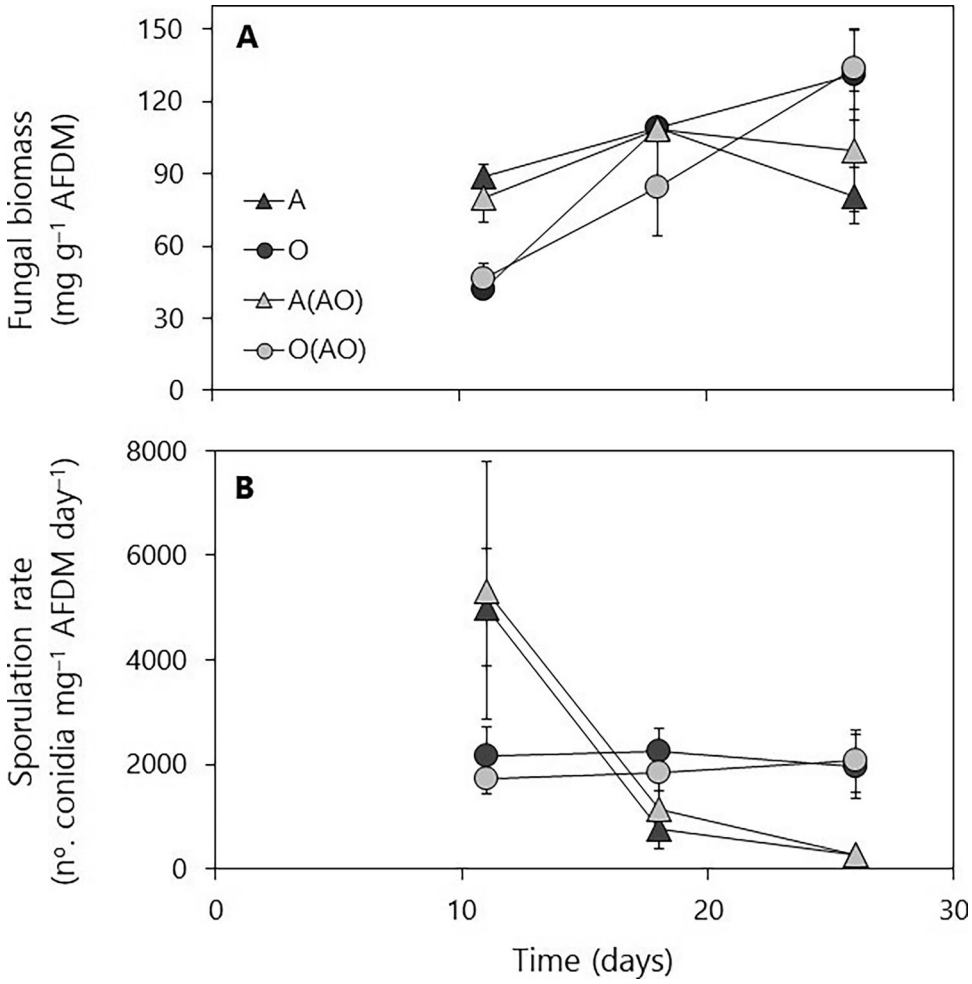

Fig. 3 Fungal biomass (**A**) and sporulation rates by aquatic hyphomycetes (**B**) (mean ± SE, $n = 3$) associated with alder and oak litter incubated individually (A and O, respectively) or in the mixture (A (AO) and O (AO), respectively) under fast current velocity conditions over time

Table 5 Summary table for two-way ANOVAs (time and litter treatment as categorical variables) on fungal biomass (log($x+1$)-transformed), aquatic hyphomycete sporulation rates (log(x)-transformed) and species richness associated with leaf litter decomposing under fast current velocity conditions, to test whether variables differed between litter species (a) when incubated individually (A vs O), (b) when incubated in the mixture (A (AO) vs O (AO)), or (c) when incubated individually or in the mixture (A vs A (AO) and O vs O (AO))

Litter treatment	A vs O			A (AO) vs O (AO)			A vs A (AO)			O vs O (AO)		
Variable	*df*	*F*	*P*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Fungal biomass												
Time	**2**	**13.76**	**0.001**	**2**	**14.38**	**< 0.001**	**2**	**3.21**	**0.083**	**2**	**36.94**	**< 0.001**
Treatment	1	2.04	0.180	1	3.38	0.092	1	0.00	0.956	1	0.09	0.765
Time × treatment	2	11.61	**0.002**	2	5.79	**0.019**	2	0.67	0.530	2	0.84	0.456
Residuals	11			11			10			12		
Sporulation rate												
Time	2	13.42	**< 0.001**	2	5.56	**0.019**	2	23.02	**< 0.001**	2	0.05	0.940
Treatment	1	10.00	**0.008**	1	3.68	0.078	1	0.01	0.919	1	0.28	0.600
Time × treatment	2	11.72	**0.001**	2	6.47	**0.012**	2	0.16	0.851	2	0.14	0.860
Residuals	12			12			12			12		
Fungal species richness												
Time	2	5.08	**0.025**	2	2.33	0.139	2	0.22	0.804	2	5.65	**0.018**
Treatment	1	5.27	**0.040**	1	3.04	0.106	1	0.22	0.646	1	0.96	0.346
Time × treatment	2	1.34	0.296	2	1.47	0.267	2	1.55	0.251	2	1.88	0.194
Residuals	12			12			12			12		

Statistical significances are highlighted in bold

Table 6 Summary table for ANOSIM on aquatic hyphomycete community composition associated with leaf litter decomposing under fast current velocity conditions, to test whether they differed between litter species (a) when incubated individually (A vs O), (b) when incubated in the mixture (A (AO) vs O (AO)), or (c) when incubated individually or in the mixture (A vs A (AO) and O vs O (AO))

Litter treatment	*R*	*p*
A vs O	0.76	< 0.001
A (AO) vs O (AO)	0.87	< 0.001
A vs A (AO)	− 0.07	0.904
O vs O (AO)	− 0.05	0.724

the decomposition recalcitrant litter in streams. This finding contributes to explain the lack of litter-mixing effects reported previously by many studies. Although this study addressed a single litter mixture, this was composed of litter from two common and often dominant riparian tree species across Europe (alder and oak) and is thus ecologically relevant. Still, extrapolations of our results to other leaf litter mixtures requires caution, as it is generally recommended when extrapolating results to contexts differing from those under which experiments were performed.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

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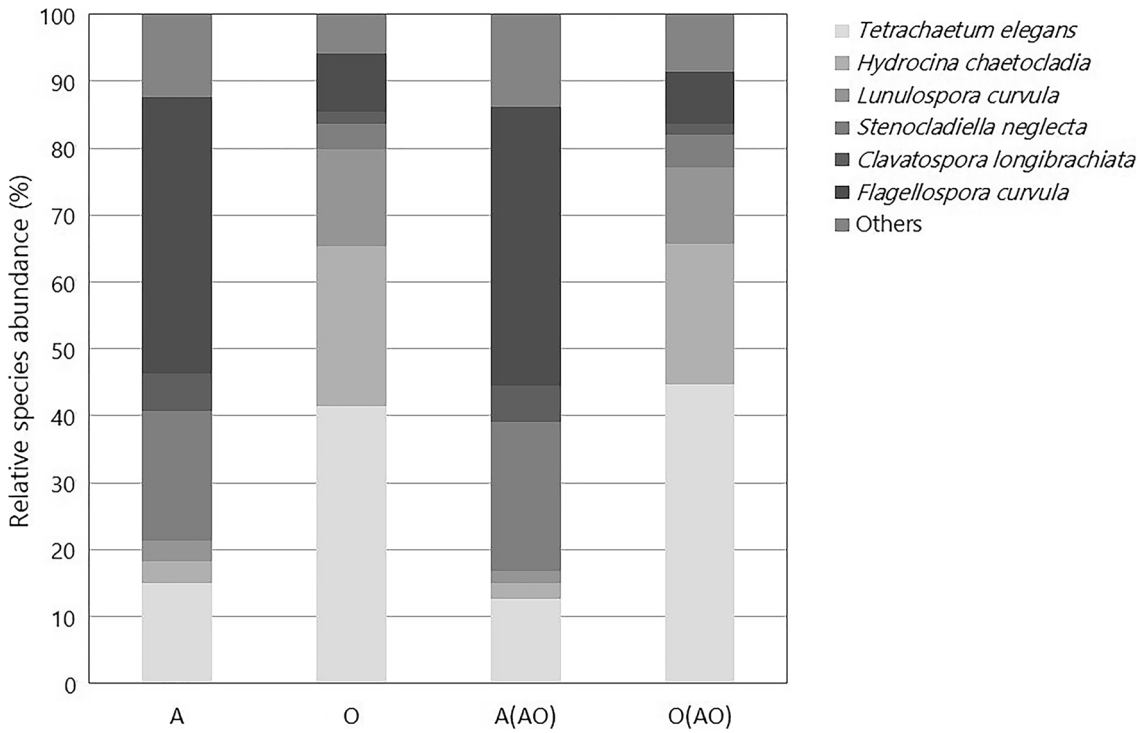

Fig. 4 Mean ($n = 9$) relative contribution of aquatic hyphomycete species to conidial production associated with alder and oak litter incubated individually (A and O, respectively) or in the mixture (A (AO) and O (AO), respectively) under fast current velocity conditions across time (three sampling dates combined). Species contributing with < 5% conidia on all treatments are shown together as "Others"

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