

Increasing inputs of invasive N-fixing *Acacia* litter decrease litter decomposition and associated microbial activity in streams

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Abstract

1. Nitrogen (N)-fixing *Acacia* species are often aggressive invaders outside their native range. When invading native riparian temperate forests, they can decrease tree species diversity, alter the quality of litter inputs to streams and increase water N concentration. Although the effects of riparian tree species diversity and nutrient enrichment on litter decomposition and associated microbial decomposers have been widely studied, their individual and combined effects remain poorly understood, especially in streams flowing through forests invaded by *Acacia* species. Here, we assessed the effects of litter diversity (species evenness) and water N concentration on the decomposition of native and *Acacia* litter, and the activity and community structure of associated microbial decomposers.
2. Litter of *Castanea sativa* (C) and *Acacia melanoxylon* (A) was enclosed in fine-mesh bags in a total of five litter evenness treatments (100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A), and immersed in a stream flowing through a native forest (native stream) and a stream flowing through a forest invaded by *Acacia* species (invaded stream).
3. Litter decomposition rates and microbial decomposer activity differed among litter evenness treatments, generally decreasing as the proportion of *A. melanoxylon* increased. When considered individually, *C. sativa* litter decomposition and associated microbial activity did not differ among treatments. For *A. melanoxylon*, decomposition rates did not differ among treatments, whereas microbial activity was generally lower in treatments with higher or even proportions of *C. sativa*.
4. Litter diversity had (small) antagonistic effects on litter decomposition in streams. However, litter treatments affected by diversity (species evenness) effects differed between streams, suggesting that effects can be modulated by water N concentration.
5. Litter decomposition rates and microbial decomposer activity were higher in the invaded than in the native stream, probably as a consequence of the higher water N concentration in the former stream. However, the magnitude of the effects was small owing to the fact that water N concentration was still in the oligotrophic range in the invaded stream.

6. Overall, our results suggest that the increasing proportion of N-fixing *Acacia* species in invaded deciduous riparian forests will affect litter decomposition rates and microbial decomposer activity, and alter aquatic hyphomycete community structure, most probably as a result of decreases in the diversity and quality of litter inputs to streams, and increases in water N concentration. However, the magnitude of the effects resulting from decreases in litter input diversity and quality (due to increases in *Acacia* contribution) into invaded streams will probably be larger than those resulting from increases in water N concentration, thus overall litter decomposition will decrease. These impacts will possibly alter nutrient cycles in aquatic food webs that depend on riparian detritus, with implications for stream functioning.

KEYWORDS

aquatic hyphomycetes, exotic species, forest invasion, litter mixture, organic-matter processing

1 | INTRODUCTION

The spread of exotic invasive plant species continues to increase worldwide, leading to biodiversity loss and impaired ecosystem functioning (Gallardo et al., 2016; Pyšek et al., 2020; Pyšek & Richardson, 2010; Vitousek et al., 1997). Riparian forests are particularly vulnerable to invasion by exotic plant species because anthropogenic (e.g., land-use practices) and natural (e.g., floods) disturbances increase the vulnerability of riparian zones to invasion and facilitate the establishment of exotic plant species (Castro-Díez & Alonso, 2017; Richardson et al., 2007). Additionally, streams can act as corridors for the dispersal of exotic plant propagules and facilitate the invasion of riparian forests along the stream network (Catford & Jansson, 2014; Čuda et al., 2017; Richardson et al., 2007).

Riparian forests are extremely important for the structure and functioning of small stream ecosystems (Jones & Swan, 2016; Tolkkinen et al., 2020; Vannote et al., 1980; Wallace et al., 1997). The riparian vegetation shapes abiotic stream conditions, e.g., by regulating water temperature and the amount of metals, sediments, nutrients and organic matter that enters into streams, provides habitat and food resources for aquatic organisms, and controls the diversity of aquatic communities (Tolkkinen et al., 2020). In small forest streams, the decomposition of organic matter inputs from the riparian vegetation is a key process by which energy and nutrients are transferred along aquatic food webs (Abelho, 2001; Petersen & Cummins, 1974; Wallace et al., 1997). Aquatic microbial decomposers (mainly aquatic hyphomycetes, but also bacteria) are the main responsible for the transfer of plant nutrients to higher trophic levels, simultaneously promoting litter mass loss by mineralising litter carbon (C), incorporating it into biomass and facilitating the release of fine particulate organic matter (Baldy et al., 2002; Gessner et al., 2007; Gulis & Suberkropp, 2003; Hieber & Gessner, 2002).

Aquatic microbial decomposers, and consequently litter decomposition, strongly depend on litter physical and chemical characteristics (e.g., Ferreira, Castela, et al., 2016; Gessner & Chauvet, 1994; Pereira & Ferreira, 2021; Pereira et al., 2016; Schindler & Gessner, 2009), and nutrient concentrations in water (e.g., Ferreira et al., 2006; Gulis & Suberkropp, 2003; Pereira et al., 2016; Woodward et al., 2012). Generally, high-quality litter (i.e., soft, high N, and low lignin and polyphenols concentrations) is colonized and decomposed faster than low-quality resources (i.e., tough, low N, and high lignin and polyphenols concentrations) (e.g., Gessner & Chauvet, 1994; Pereira & Ferreira, 2021; Pereira et al., 2016, 2017; Schindler & Gessner, 2009). Additionally, low to moderate increases in water nutrient concentration [e.g., N, phosphorus (P)] in oligotrophic streams can accelerate litter decomposition due to a stimulation of microbial decomposer activity (e.g., Ferreira et al., 2006; Gulis et al., 2006; Gulis & Suberkropp, 2003; Pereira et al., 2016, 2017). The effects of nutrient enrichment in stream water are generally stronger for substrates with low nutrient concentrations, where microbes are nutrient-limited (Ferreira et al., 2006; Gulis et al., 2006; Gulis & Suberkropp, 2003; Kominoski et al., 2015). Also, nutrient enrichment may stimulate microbial activity in substrates with low concentration of low-quality C, where microbes are not C-limited (Jabiol et al., 2019).

Acacia species are leguminous shrubs and trees native to Australia that have been introduced into several regions around the world since the late 1700s (Carruthers et al., 2011). Currently, 24 *Acacia* species are considered among the most problematic exotic invaders in many European, American and African countries (Richardson & Rejmánek, 2011). *Acacia* species are very aggressive and fast-growing invaders that progressively eliminate the native vegetation of the recipient ecosystem (Le Maitre et al., 2011; Souza-Alonso et al., 2017), creating very dense and homogeneous stands of trees. Therefore, the diversity and richness of organic matter

inputs to streams will probably decrease as the proportion of *Acacia* trees increases in invaded deciduous riparian forests. Moreover, the quality of the litter entering streams will most likely change, since the chemical and physical characteristics of *Acacia* litter (e.g., *Acacia dealbata* Link., *Acacia melanoxylon* R. Br.) can differ from those of the native trees usually present in the riparian vegetation of temperate streams [e.g., *Alnus glutinosa* (L.) Gaertn., *Quercus robur* L.] (Pereira & Ferreira, 2021). Additionally, since *Acacia* species can fix atmospheric N, through their association with root-nodule bacteria (e.g., *Bradyrhizobium* spp.; Souza-Alonso et al., 2017), water N concentration will most likely increase, as observed recently in streams with riparian forests invaded by *Acacia* species (Pereira et al., 2021), or in streams flowing through forests composed by other N-fixing species (Compton et al., 2003; Goldstein et al., 2009; Shaftel et al., 2012; Singh et al., 2019; Stewart et al., 2019; Wiegner et al., 2013).

Since decreases in riparian tree species diversity and increases in stream water N concentration are expected to occur simultaneously as the proportion of N-fixing *Acacia* species in native deciduous riparian forests increases, it becomes important to assess the effects of litter diversity (e.g., species evenness) and water N concentration on litter decomposition, and the activity and community structure of microbial decomposers, if we want to better understand and predict the response of stream ecosystems to *Acacia* species invasion. Previous studies have found that, in streams, litter diversity effects on litter decomposition are generally nonadditive (e.g., 2016 Kominoski et al., 2007; Lecerf et al., 2011; Lima-Fernandes et al., 2015; Rosemond et al., 2010; Swan & Palmer, 2004; Swan et al., 2009), and may occur because: (a) microbial decomposers colonise and decompose faster high-quality species in the presence of low-quality litter (Lecerf et al., 2011; Rosemond et al., 2010); (b) fungal mycelium growth may allow nutrient transfer between high- and low-quality litter species, reducing their heterogeneity for microbes (Gessner et al., 2010; Lecerf et al., 2011; Rosemond et al., 2010); and/or (c) secondary compounds that inhibit fungal activity can be leached from low-quality resources to other litter species in the mixture (Gessner et al., 2010). Nevertheless, in nutrient-enriched streams, nonadditive litter diversity effects can be suppressed by the high concentration of nutrients (i.e., N) in water, due to a homogenization of resource quality to aquatic decomposers (Lima-Fernandes et al., 2015; Rosemond et al., 2010). Although the individual and combined effects of litter diversity and nutrient concentrations in water have been assessed previously on stream communities and processes, their effects were never addressed in the context of biological invasions, where the realistic conditions (e.g., physical, chemical and biological) of streams flowing through forests invaded by N-fixing *Acacia* species were taken into account. In this study, we assessed the individual and combined effects of litter species evenness and water N concentration in the decomposition of native *Castanea sativa* Miller and invasive *A. melanoxylon* litter, and the activity and community structure of microbial decomposers, in a stream flowing through a native deciduous forest (native stream) and a stream flowing through a forest invaded by *Acacia* species (invaded stream). We predicted that: (a) litter decomposition

and associated microbial decomposer activity would decrease as the proportion of *A. melanoxylon* litter increased across litter evenness treatments, since *A. melanoxylon* is a poor-quality resource for aquatic microbes; (b) in the native stream, litter mixtures would decompose slower than predicted from the decomposition of the component litter species incubated individually (nonadditive effect), due to differences in the physical and chemical characteristics of the native and invasive species; (c) in the invaded stream, litter diversity effects would be modulated by the higher water N concentration, because microbes would not be N-limited, and would obtain their nutrient requirements directly from the stream water; (d) litter decomposition and microbial activity would be faster in the invaded than in the native stream, due to a stimulation of microbial activity by the increased N concentration in the former stream; and (e) the community structure of aquatic hyphomycetes would differ among litter evenness treatments due to increases in the contribution of recalcitrant *A. melanoxylon* litter to litter mixtures, and between the native and the invaded stream due to higher water N concentration in the latter stream.

2 | METHODS

2.1 | Study streams

This study took place in two streams located in Serra da Lousã, central Portugal, which differ in the composition of the riparian vegetation. Cerdeira stream (40°05'23.1"N, 8°12'05.0"W, 529 m a.s.l.; native stream) drains a 337 ha basin with 20% native mixed deciduous forests, 23% conifer plantations, 54% bushes and 0.12% *Acacia* species stands; in the study area it flows through a native mixed deciduous forest mainly composed of *C. sativa* and *Q. robur* trees, with 1% *Acacia* species in the riparian area (Pereira et al., 2021). Sotão stream (40°07'54.1"N, 8°09'08.3"W, 373 m a.s.l.; invaded stream) drains a 114 ha basin with 6% native mixed deciduous forests, 26% *Eucalyptus globulus* Miller plantations, 45% conifer plantations and 15% *Acacia* species stands; in the study area it flows through a forest heavily invaded by exotic N-fixing *A. dealbata* and *A. melanoxylon* (94% in the riparian area) (Pereira et al., 2021). Both stream basins have low human and agricultural activity (0%–1.2% of urban areas and 0%–0.5% of agricultural areas; Pereira et al., 2021). Streams are <4 m wide, and substrate is composed mainly of schist gravel and cobbles. We considered only one stream of each type as the thorough assessment of microbial activities associated with the decomposing litter would not be possible for a larger number of streams (see Sections 2.2–2.5). However, the selected streams are representative of their stream type, as found in a previous study where we used multiple native and invaded streams, including the streams used here (Pereira et al., 2021).

Water temperature was recorded hourly during the experiment with data loggers (Hobo Pendant UA-001-08; Onset Computer Corp., Bourne, MA, USA), while dissolved O₂ concentration, pH and conductivity were measured in situ with field probes (WTW,

Weilheim, Germany). Additionally, water from each stream was filtered (glass fibre filters, 47 mm diameter, 0.7- μ m pore size; Whatman GF/F, GE Healthcare UK Ltd, Little Chalfont, UK) into acid-washed plastic bottles, transported in a cooler to the laboratory and used to determine nitrites, nitrates and ammonia concentrations (colorimetric method, AA3 Bran + Luebbe autoanalyzer; SEAL Analytical, Norderstedt, Germany).

2.2 | Litter species and litter decomposition

Leaves of native *C. sativa* and phyllodes (i.e., leaf-like petioles) of invasive *A. melanoxylo*n were collected in Autumn 2016, immediately after abscission, in Serra do Açor (Margarça forest) and Serra da Lousã, respectively, air-dried at room temperature and stored in the dark until used. In central Portugal, *C. sativa* trees are commonly found in the riparian vegetation of streams flowing through native deciduous forests, while *A. melanoxylo*n is present in the riparian vegetation of streams flowing through forests invaded with N-fixing *Acacia* species. Three sets of air-dried litter from each species were oven-dried at 105°C for 48 hr and ground to a fine powder (<0.5 mm size, ZM 100 Ultra Centrifugal Mill; Retsch, Haan, Germany). The powder was oven-dried at 105°C for 48 hr, and used to determine the initial lignin (Goering & Van Soest, 1970), polyphenols and P (Bärlocher et al., 2020), C and N (CNH auto analyzer IRMS Thermo Delta V advantage with a Flash EA, 1112 series; Thermo Fisher Scientific Inc., Waltham, MA, USA) concentrations. Concentrations were expressed as percentage of dry mass (DM). Carbon:nutrients (i.e., C:N and C:P) and N:P molar ratios were calculated. Litter toughness was determined using a penetrometer (0.49-mm diameter iron rod) as the mass (g) required to punch a hole in the litter (Bärlocher et al., 2020) and specific litter area was determined as the ratio of area to mass (mm²/mg) for 12-mm discs after being oven-dried at 105°C for 24 hr.

Air-dried *C. sativa* (C) and *A. melanoxylo*n (A) litter were enclosed individually or in two-species mixtures in fine-mesh bags (0.5-mm mesh size) in a total of five litter evenness treatments: 100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A, which simulated an increase in the contribution of *Acacia spp.* litter to streambeds of streams flowing through invaded forests. Twelve litter bags per litter evenness treatment, containing 3 g litter each (distributed proportionally among the component species according to the litter evenness treatment), were immersed in each stream on 5 December 2016 (12 litter bags \times five litter evenness treatments \times two streams = 120 litter bags).

After 7, 21, 42 and 77 days of stream immersion, three litter bags of each litter evenness treatment were retrieved from each stream, placed individually in zip lock bags and transported in a cooler to the laboratory. Litter was gently rinsed with distilled water over a 500- μ m mesh sieve to remove sediments and retain small litter fragments. Litter species from mixtures were processed individually. Two sets of five litter discs (12-mm diameter) were cut, when possible, from individual litter of *C. sativa* (avoiding

major veins) or *A. melanoxylo*n, and used to determine fungal biomass and induce conidia production by aquatic hyphomycetes (see Sections 2.3 and 2.4).

The remaining litter was oven-dried at 105°C for 48 hr, weighed to determine DM remaining and ground to a fine powder. A subsample of litter powder was oven-dried at 105°C for 24 hr, weighed, ignited at 500°C for 4 hr, and reweighed to determine the ash fraction needed to convert DM of the sample into ash-free dry mass (AFDM). Results were expressed as percentage of initial AFDM, considering the mass of the discs extracted (see Sections 2.3 and 2.4). Five additional litter bags of each litter species were prepared as described above and used to estimate the initial air-DM to initial AFDM conversion factor.

2.3 | Fungal biomass

One set of five litter discs was used to determine ergosterol concentration as a measure of fungal biomass (Bärlocher et al., 2020). Fungal biomass was determined on days 21, 42 and 77, and litter species from mixtures were processed individually. Litter discs, previously stored at -20°C, were lyophilised overnight and weighed to determine DM. Lipids were extracted by heating litter discs in 8 g/L of KOH/methanol at 80°C for 30 min, purified by solid phase extraction (Sep-Pak Vac RC tC18 cartridges; Waters Corp., Milford, MA, USA), and quantified at 282 nm by high performance liquid chromatography (HPLC, DX-120; Dionex, Sunnyvale, CA, USA) (Bärlocher et al., 2020). Ergosterol was converted to fungal biomass using a conversion factor of 5.5 μ g ergosterol/mg fungal DM (Gessner & Chauvet, 1993). Fungal biomass was expressed as mg fungal DM/g litter AFDM.

2.4 | Aquatic hyphomycetes conidia production

The other set of five litter discs of each litter species was used to determine conidia production by aquatic hyphomycetes. Litter species from mixtures were processed individually. Litter discs were incubated in 100-ml Erlenmeyer flasks with 25 ml filtered stream water. Flasks were incubated for 48 hr on a shaker, at 18°C, under a 12 hr : 12 hr, light : dark photoperiod. Conidia suspensions were placed in 50-ml Falcon tubes, fixed with 2 ml of 37% formaldehyde solution, and stored in the dark until conidia identification and counting. Litter discs were oven-dried at 105°C for 48 hr, weighed to determine DM, ignited at 500°C for 4 hr, and reweighed to determine AFDM.

When preparing slides, conidia suspensions were mixed with 100 μ l Triton X-100 solution (0.5%), filtered through cellulose nitrate filters (diameter 25 mm, 5- μ m pore size; Sartorius Stedim Biotech GmbH, Goettingen, Germany) and filters were stained with trypan blue (0.05%) in lactic acid (60%). Spores were identified and counted under a stereo microscope at 200 \times magnification (DM1000; Leica, Wetzlar, Germany) (Bärlocher et al., 2020). Sporulation rates by

aquatic hyphomycetes were determined as the number of released conidia/mg litter AFDM/day and aquatic hyphomycetes species richness as the number of species/sample.

2.5 | Statistical analysis

Water characteristics were compared between the native and the invaded stream, and litter initial physical characteristics (i.e., toughness and SLA) were compared between the *C. sativa* and *A. melanoxylo*n litter by one-way ANOVA (Zar, 2010). Litter toughness and SLA were only compared between *C. sativa* and *A. melanoxylo*n since it was not possible to determine these parameters for litter mixtures. Initial chemical characteristics (i.e., lignin, polyphenols, C, N and P concentrations, and C:N, C:P and N:P molar ratios) of litter mixtures were determined using the initial characteristics of each litter species, taking into account their proportion in treatments. Litter initial chemical characteristics were compared among litter treatments by one-way ANOVAs, followed by Tukey's honestly significant difference (HSD) tests, when significant differences were found (Zar, 2010). Data were transformed [conductivity was $\log(x + 1)$ transformed, and water temperature, NH_4^+ -N concentration, toughness, SLA, and polyphenols and P concentrations, and N:P (molar) were Box-Cox transformed] when necessary to meet the normality (Kolmogorov-Smirnov or D'Agostino and Pearson test) and homoscedasticity (Levene's or Bartlett's test) assumptions of ANOVA (Zar, 2010).

Observed decomposition rates (k , /d) were calculated for each sample assuming the negative exponential model: $M_f = M_i e^{-kt}$, where M_f is the final AFDM (g), M_i is the initial AFDM (g) and t is time in days. To consider the temperature differences observed between the native and the invaded stream, decomposition rates of each sample were estimated on a degree-day basis (k , /dd), by replacing time with the cumulative mean daily temperatures by each sampling day. Expected AFDM remaining of litter mixtures (75%C + 25%A, 50%C + 50%A and 25%C + 75%A) was calculated for each sample assuming a double exponential model: $M_{(C+A)t} = M_C e^{-k_C t} + M_A e^{-k_A t}$, where $M_{(C+A)t}$ is the mass remaining in the mixture at time t , M_C and M_A are the initial mass of *C. sativa* and *A. melanoxylo*n litter in the mixture, respectively, and k_C and k_A are the decomposition rates of *C. sativa* and *A. melanoxylo*n litter in single litter treatments, respectively (Ostrofsky, 2007). Expected decomposition rates of each sample of litter mixtures were estimated on a degree-day basis. Differences between expected and observed decomposition rates (k , /dd, Box-Cox transformed) among sampling dates were assessed for each litter mixture and stream by two-way ANOVAs (Zar, 2010).

Litter decomposition rates (k , /dd), fungal biomass, and aquatic hyphomycetes sporulation rates and species richness of litter evenness treatments, and of *C. sativa* and *A. melanoxylo*n litter from litter evenness treatments, were compared between streams, among litter treatments and time by three-way univariate PERMANOVA (Anderson et al., 2008) since data failed to meet the assumptions of ANOVA. Although PERMANOVA is used mostly to perform multivariate analysis, when ran on Euclidean distance it holds the

same F -statistic value and the equivalent p -value (obtained by permutations) as ANOVA analysis (Anderson et al., 2008). Therefore, PERMANOVA can be used as a nonparametric test to perform univariate ANOVAs (e.g., Ainley & Bishop, 2015; Caspi et al., 2019; Orozco-Aceves et al., 2015; Seena et al., 2017; Seena et al., 2019). Univariate PERMANOVAs were run on Euclidean distance, with residuals permuted under a reduced model of 9,999 permutations, followed by pairwise comparison tests when significant differences were found (Anderson et al., 2008).

The structure of aquatic hyphomycete communities (based on the conidia production by each aquatic hyphomycete species) was assessed by non-metric multidimensional scaling (nMDS) analysis. Samples of the 100%A treatment collected after 7 and 21 days of immersion in the native stream and collected after 7 days of immersion in the invaded stream were not considered in the analysis, since aquatic hyphomycete sporulation rates were extremely low, and affected the ordination of treatments in the analysis. Communities were compared between streams, and among litter treatments and time by three-way multivariate PERMANOVA, followed by pairwise comparison tests, when significant differences were found (Anderson et al., 2008). Additionally, PERMDISP analyses were performed to test the homogeneity of multivariate dispersions within groups (Anderson et al., 2008). A similarity of percentage analysis (SIMPER) was also performed for each stream to identify the aquatic hyphomycete species most responsible for the dissimilarities observed between litter treatments. Before all analyses, aquatic hyphomycete sporulation rates were transformed [$\log(x + 1)$] and converted into a Bray-Curtis similarity matrix.

Normality and homoscedasticity analyses, and ANOVAs were performed on STATISTICA 8 software (StatSoft Inc., Tulsa, OK, USA). Univariate and multivariate PERMANOVAs, pairwise comparison tests, nMDS, PERMDISP, and SIMPER analyses were performed on PRIMER 6 (v6.1.16) and PERMANOVA+ (v1.0.6; Primer-E Ltd, Plymouth, UK).

3 | RESULTS

3.1 | Water characteristics

In both streams, water was cold, circumneutral, well-oxygenated, and had low conductivity and nutrient concentrations (Table 1). Water temperature, conductivity, and NO_3^- -N and dissolved inorganic N (DIN) concentrations were significantly higher in the invaded than in the native stream (one-way ANOVA, $p \leq 0.001$) (Table S1). No significant differences were found in pH, and dissolved O_2 , NO_2^- -N, and NH_4^+ -N concentrations between the native and the invaded stream (one-way ANOVA, $p \geq 0.072$) (Table S1).

3.2 | Initial litter characteristics

Litter toughness was significantly higher, and SLA was significantly lower in *A. melanoxylo*n than in *C. sativa* litter (one-way ANOVA,

$p < 0.001$) (Tables 2 and S2). Lignin, N and P concentrations, and C:P and N:P ratios differed significantly among litter treatments (one-way ANOVA, $p \leq 0.007$) (Table S2). Generally, lignin and N concentrations, and C:P and N:P ratios increased as the proportion of *A. melanoxylon* increased among litter treatments, while P concentration varied in the opposite direction (Table 2). No significant differences were found in initial polyphenols and C concentrations, and C:N ratios between litter treatments (one-way ANOVA, $p \geq 0.065$) (Table S2).

3.3 | Litter decomposition

Litter mass remaining decreased exponentially over the incubation period and after 77 days of stream immersion, litter mass remaining in the litter evenness treatments varied between 48% and 74% in

TABLE 1 Water characteristics of the native (Cerdeira) and the invaded (Sotão) stream located in Serra da Lousã (central Portugal) during the litter decomposition experiment

Water characteristics	Native stream	Invaded stream
Temperature (°C)*	8.35 ± 0.19	9.51 ± 0.19
Dissolved O ₂ (mg/L)	10.93 ± 0.31	10.94 ± 0.30
pH	7.37 ± 0.05	7.23 ± 0.04
Conductivity (µS/cm)*	33.33 ± 1.20	59.50 ± 1.23
NO ₂ ⁻ - N (µg/L)	0.86 ± 0.19	1.35 ± 0.29
NO ₃ ⁻ - N (µg/L)*	5.51 ± 0.91	40.23 ± 4.49
NH ₄ ⁺ - N (µg/L)	19.99 ± 2.50	43.86 ± 11.86
DIN (µg/L)*	26.36 ± 3.11	85.45 ± 12.15

Note: Values are means ± SE ($n = 5-6$, except for temperature where $n = 77$). Asterisks indicate significant differences between streams (one-way ANOVAs, $p < 0.050$).

Abbreviation: DIN, dissolved inorganic nitrogen.

TABLE 2 Initial physical characteristics of *Castanea sativa* (C) and *Acacia melanoxylon* (A) litter, and chemical characteristics of the litter evenness treatments used in the decomposition experiment

Litter variables	100%C	75%C + 25%A	50%C + 50%A	25%C + 75%A	100%A
Toughness (g)	70.1 ± 6.4 ^a	-	-	-	229.5 ± 8.1 ^b
SLA (mm ² /mg)	23.9 ± 1.8 ^a	-	-	-	8.9 ± 0.5 ^b
Lignin (% DM)	27.9 ± 2.0 ^a	30.5 ± 1.2 ^{ab}	33.0 ± 0.5 ^{abc}	35.5 ± 0.5 ^{bc}	38.1 ± 1.2 ^c
Polyphenols (% DM)	5.2 ± 2.3	5.1 ± 1.8	5.0 ± 1.3	4.9 ± 0.8	4.8 ± 0.4
C (% DM)	48.4 ± 0.6	48.7 ± 0.5	48.9 ± 0.4	49.2 ± 0.4	49.4 ± 0.3
N (% DM)	0.9 ± 0.1 ^a	1.1 ± 0.1 ^{ab}	1.2 ± 0.1 ^{abc}	1.4 ± 0.1 ^{bc}	1.5 ± 0.1 ^c
P (% DM)	0.233 ± 0.023 ^a	0.177 ± 0.018 ^{ab}	0.121 ± 0.012 ^b	0.065 ± 0.006 ^c	0.009 ± 0.001 ^d
C:N (molar)	65.0 ± 9.7	58.2 ± 7.3	51.4 ± 5.0	44.7 ± 2.9	37.9 ± 1.9
C:P (molar)	548 ± 56 ^a	4,052 ± 326 ^b	7,556 ± 614 ^c	11,060 ± 904 ^d	14,564 ± 1,193 ^e
N:P (molar)	9 ± 2 ^a	104 ± 14 ^{ab}	199 ± 26 ^{bc}	294 ± 39 ^{cd}	389 ± 52 ^d

Note: Values are means ± SE ($n = 10$ for physical variables and $n = 3$ for chemical variables). Different letters indicate significant differences among litter treatments (one-way ANOVAs followed by Tukey's HSD, $p < 0.050$).

Abbreviations: C, carbon; N, nitrogen; P, phosphorus; SLA, specific leaf area.

the native stream and between 36% and 68% in the invaded stream (Figure 1a,d). When considering individual litter species, *C. sativa* litter mass remaining varied between 48% and 52% in the native stream and between 34% and 40% in the invaded stream (Figure 1b,e), while *A. melanoxylon* litter mass remaining varied between 74% and 77% in the native stream and between 68% and 70% in the invaded stream (Figure 1c,f).

Decomposition rates in the litter evenness treatments differed significantly between streams and among litter treatments, and there was a significant interaction between both factors (three-way univariate PERMANOVA, $p < 0.001$) (Table S3). Decomposition rates were significantly higher in the invaded than in the native stream for all litter treatments (pairwise tests, $p < 0.001$), except for 100%A that did not differ between streams ($p = 0.079$), and decreased as the proportion of *A. melanoxylon* increased in litter treatments (100 %C > 75%C + 25%A > 50%C + 50%A > 25%C + 75%A > 100%A, in both streams; $p \leq 0.001$) (Figure 2a).

When considering individual litter species, decomposition rates of *C. sativa* and *A. melanoxylon* litter were significantly higher in the invaded than in the native stream (three-way univariate PERMANOVA, $p \leq 0.002$), but did not differ among litter treatments, neither was there a significant interaction between stream and litter treatment ($p \geq 0.304$) (Figure 2b,c; Table S3).

In the native stream, decomposition rates of 50%C + 50%A and 25%C + 75%A litter treatments were 5%–14% lower (across sampling dates) than expected from the decomposition rates of the component litter species incubated individually, while in the invaded stream, decomposition rates of 75%C + 25%A and 50%C + 50%A litter treatments were 2%–27% lower (across sampling dates) than expected, with differences between observed and expected rates being significant (two-way ANOVAs, $p \leq 0.007$), suggesting nonadditive (antagonistic) effects of litter diversity on litter decomposition (Tables 3 and S4). For the 75%C + 25%A litter treatment in the native stream and the 25%C + 75%A in the invaded stream, no significant

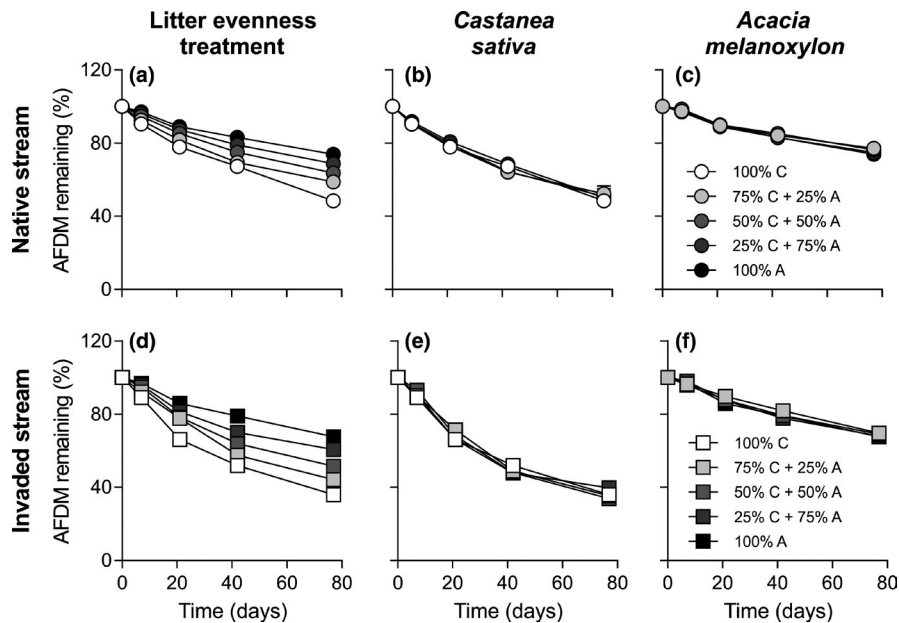


FIGURE 1 Ash-free dry mass (AFDM) remaining of litter evenness treatments (100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A; C, *Castanea sativa*; A, *Acacia melanoxylon*) (a, d), and of *C. sativa* (b, e) and *A. melanoxylon* (c, f) litter from litter evenness treatments incubated in the native and in the invaded stream located in Serra da Lousã (central Portugal), over 77 days. Values are means \pm SE ($n = 3$); SE bars are smaller than the symbols

differences were found between the observed and expected decomposition rates (two-way ANOVAs, $p \geq 0.227$) (Tables 3 and S4).

3.4 | Fungal biomass

Fungal biomass in the litter evenness treatments increased until a peak was attained in treatments with $\geq 50\%$ *C. sativa* or it increased over the incubation period in treatments with higher proportion of *A. melanoxylon* than of *C. sativa* litter, and maximum fungal biomass varied between 22 and 55 mg/g AFDM in the native stream and between 39 and 95 mg/g AFDM in the invaded stream (Figure 3a,d). When considering individual litter species, *C. sativa* fungal biomass increased until a peak was attained by day 21 in the invaded stream and day 42 in the native stream, and maximum fungal biomass varied between 50 and 67 mg/g AFDM in the native stream and between 82 and 95 mg/g AFDM in the invaded stream (Figure 3b,e). *Acacia melanoxylon* fungal biomass increased over the incubation period, and maximum fungal biomass varied between 10 and 23 mg/g AFDM in the native and between 35 and 41 mg/g AFDM in the invaded stream (Figure 3c,f).

Fungal biomass in litter evenness treatments differed significantly between streams and among litter treatments, with a significant interaction between both factors (three-way univariate PERMANOVA, $p \leq 0.005$) (Table S5). Fungal biomass was significantly higher in the invaded than in the native stream for all litter treatments (pairwise tests, $p \leq 0.009$) (Figure 3a,d). In the native stream, fungal biomass was significantly higher in the 100%C litter treatment than in all others (pairwise tests, $p \leq 0.001$), was significantly lower in the 100%A litter treatment than in all others ($p \leq 0.008$) and did not differ among litter mixture treatments ($p \geq 0.226$) (Figure 3a). In the invaded stream, fungal biomass decreased as the proportion of *A. melanoxylon* increased (100%C > 75%C + 25%A > 50%C + 50%A > 25%C + 75%A > 100%A; pairwise tests, $p \leq 0.040$) (Figure 3d).

When considering individual litter species, fungal biomass in *C. sativa* litter differed significantly between streams and among litter treatments (three-way univariate PERMANOVA, $p \leq 0.017$), but there was no significant interaction between both factors ($p = 0.082$) (Table S5). Fungal biomass was significantly higher in the invaded than in the native stream (three-way univariate PERMANOVA, $p < 0.001$) (Table S5), and was significantly lower in 100%C and 75%C + 25%A than in the 25%C + 75%A litter treatment (pairwise tests, $p \leq 0.036$) (Figure 3b,e). Fungal biomass in *A. melanoxylon* litter was significantly higher in the invaded than in the native stream (three-way univariate PERMANOVA, $p < 0.001$), but did not differ among litter treatments; neither was there a significant interaction between stream and litter treatment ($p \geq 0.169$) (Table S5; Figure 3c,f).

3.5 | Aquatic hyphomycetes sporulation

The dynamics of aquatic hyphomycete sporulation rates were similar to those of fungal biomass. Sporulation rates in the litter evenness treatments increased until a peak was attained, except for the litter treatment with the highest proportion of *A. melanoxylon* in the native stream, where sporulation rates increased over the incubation period (Figure 4a,d). Maximum sporulation rates varied between 273 and 2,307 conidia/mg AFDM/day in the native stream and between 1,442 and 7,226 conidia/mg AFDM/day in the invaded stream (Figure 4a,d). When considering individual litter species, sporulation rates in *C. sativa* litter increased until a peak was attained by day 21 in both streams, followed by a sharper decrease in the invaded than in the native stream, and maximum sporulation rates varied between 1,076 and 2,942 conidia/mg AFDM/day in the native stream and between 5,830 and 7,226 conidia/mg AFDM/day in the invaded stream (Figure 4b,e). Sporulation rates in *A. melanoxylon* litter increased over the incubation period, except in litter treatments with

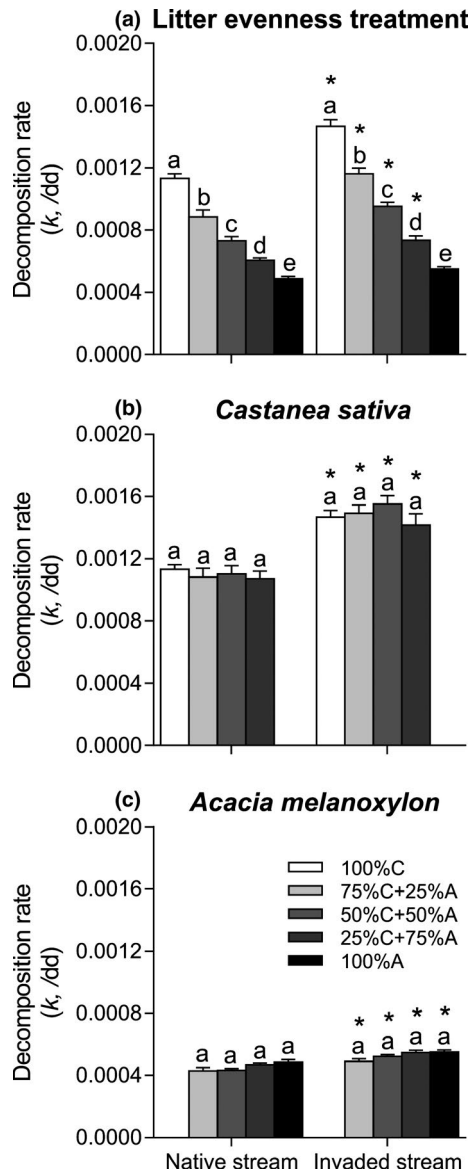


FIGURE 2 Decomposition rate of litter evenness treatments (100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A; C, *Castanea sativa*; A, *Acacia melanoxylon*) (a), and of *C. sativa* (b) and *A. melanoxylon* (c) litter from litter evenness treatments incubated in the native and in the invaded stream located in Serra da Lousã (central Portugal), over 77 days. Values are means \pm SE ($n = 3$). Comparisons between streams and among litter treatments were done by univariate PERMANOVAs; * indicate significant differences between streams for a given litter treatment and different letters indicate significant differences among litter treatments for a given stream (pairwise comparison tests, $p < 0.050$)

$\geq 50\%$ *A. melanoxylon* in the invaded stream, where a small peak was attained by day 42, and maximum sporulation rates varied between 175 and 359 conidia/mg AFDM/day in the native stream and between 630 and 1,442 conidia/mg AFDM/day in the invaded stream (Figure 4c,f).

Aquatic hyphomycete sporulation rates in the litter evenness treatments differed significantly between streams and among litter treatments (three-way univariate PERMANOVA, $p < 0.001$), but no

significant interaction was found between both factors ($p = 0.350$) (Table S6). Sporulation rates were significantly higher in the invaded than in the native stream (three-way univariate PERMANOVA, $p < 0.001$) (Table S6), and significantly decreased as the proportion of *A. melanoxylon* litter increased (100%C ~ 75%C + 25%A > 50%C + 50%A > 25%C + 75%A > 100%A; pairwise tests, $p \leq 0.007$) (Figure 4a,d).

When considering individual litter species, sporulation rates in *C. sativa* litter were significantly higher in the invaded than in the native stream (three-way univariate PERMANOVA, $p < 0.001$), but did not differ among litter treatments nor there was a significant interaction between both factors ($p \geq 0.502$) (Table S6; Figure 4b,e). Sporulation rates in *A. melanoxylon* litter differed significantly between streams, among litter treatments and there was a significant interaction between both factors (three-way univariate PERMANOVA, $p \leq 0.001$) (Table S6). Sporulation rates were significantly higher in the invaded than in the native stream for all litter treatments (pairwise tests, $p < 0.001$) (Figure 4c,f). In the native stream, sporulation rates were significantly lower in the 25%C + 75%A than in 75%C + 25%A and 50%C + 50%A litter treatments (pairwise tests, $p \leq 0.026$), while in the invaded stream, sporulation rates were significantly lower in the 75%C + 25%A than in 50%C + 50%A, 25%C + 75%A and 100%A litter treatments ($p \leq 0.003$) (Figure 4c,f).

3.6 | Aquatic hyphomycetes species richness and community structure

Aquatic hyphomycete species richness in the litter evenness treatments generally increased until a plateau was reached by day 21 (Figure 5a,d), with total number of species per treatment varying between 15 and 22 in the native stream and between 13 and 19 in the invaded stream (Table S7). When considering individual litter species, the colonisation dynamics in *C. sativa* litter was similar to that of litter treatments (Figure 5b,e), with the total number of species varying between 19 and 20 species in the native stream and between 15 and 18 species in the invaded stream (Table S7). Colonisation dynamics in *A. melanoxylon* litter was similar to the above in the invaded stream but increased over time in the native stream (Figure 5c,f), with the total number of species varying between 14 and 16 in the native stream and between 13 and 14 species in the invaded stream (Table S7).

Aquatic hyphomycete species richness per sampling date in litter evenness treatments did not differ significantly between streams (three-way univariate PERMANOVA, $p = 0.144$), but differed among litter treatments and there was a significant interaction between both factors ($p \leq 0.001$) (Table S8). In the 50%C + 50%A litter treatment, species richness was significantly higher in the native than in the invaded stream (pairwise test, $p = 0.012$), and in the 100%A it was significantly higher in the invaded than in the native stream ($p = 0.038$) (Figure 5a,d). Species richness was significantly lower in the 100%C than in 75%C + 25%A and 50%C + 50%A litter treatments in the native stream (pairwise test, $p \leq 0.022$), and 75%C + 25%A and 25%C + 75%A litter treatments in the invaded stream ($p \leq 0.014$),

TABLE 3 Observed and expected litter decomposition rates (k , /dd) of litter mixture treatments (75%C + 25%A, 50%C + 50%A, 25%C + 75%A; C, *Castanea sativa*; A, *Acacia melanoxylon*) incubated in the native and in the invaded stream located in Serra da Lousã (central of Portugal), over 77 days

Litter mixture	Time (days)	Observed	Expected	Obs/Exp	Mean Obs/Exp	SE
Native						
75%C + 25%A	7	0.0010	0.0011	0.96	0.96	0.019
	21	0.0010	0.0011	0.94		
	42	0.0010	0.0010	1.01		
	77	0.0009	0.0010	0.92		
50%C + 50%A*	7	0.0007	0.0009	0.86	0.91	0.019
	21	0.0008	0.0009	0.89		
	42	0.0008	0.0008	0.95		
	77	0.0007	0.0008	0.92		
25%C + 75%A*	7	0.0005	0.0006	0.86	0.94	0.035
	21	0.0007	0.0007	0.93		
	42	0.0007	0.0006	1.03		
	77	0.0006	0.0006	0.95		
Invaded						
75%C + 25%A*	7	0.0010	0.0011	0.88	0.89	0.040
	21	0.0011	0.0014	0.78		
	42	0.0013	0.0013	0.95		
	77	0.0012	0.0012	0.95		
50%C + 50%A*	7	0.0006	0.0009	0.73	0.90	0.061
	21	0.0010	0.0011	0.91		
	42	0.0011	0.0011	1.00		
	77	0.0010	0.0010	0.98		
25%C + 75%A	7	0.0005	0.0006	0.81	0.95	0.052
	21	0.0009	0.0009	1.00		
	42	0.0009	0.0008	1.05		
	77	0.0007	0.0008	0.97		

Note: The ratio between observed and expected (Obs/Exp) decomposition rates is also shown. Asterisks indicate significant differences between observed and expected decomposition rates (two-way ANOVAs, $p < 0.050$).

and was significantly lower in the 100%A litter treatment than in all the others, in both streams ($p < 0.001$) (Figure 5a,d).

When considering individual litter species, aquatic hyphomycete species richness per sampling date in *C. sativa* litter was significantly higher in the native than in the invaded stream (three-way univariate PERMANOVA, $p = 0.045$) (Figure 5b,e), while species richness in *A. melanoxylon* litter was significantly higher in the invaded than in the native stream ($p = 0.001$) (Figure 5c,f; Table S8). In both litter species, species richness did not differ among litter treatments, nor was there a significant interaction between stream and litter treatment (three-way univariate PERMANOVA, $p \geq 0.410$) (Table S8).

The aquatic hyphomycete species contributing most to conidia production, across litter treatments and streams, were *Anguillospora filiformis* Greath., *Articulospora tetracladia* Ingold, *Dimorphospora foliicola* Tubaki, *Hydrocina chaetoclada* Scheuer, *Tetrachaetum elegans* Ingold, and *Triscelophorus acuminatus* Nawawi (Table S7). Considering the most abundant aquatic hyphomycete species, *A. filiformis* was

likewise abundant in *C. sativa* (15%–25% of total conidial production across treatments) and *A. melanoxylon* litter (17%–22%, except for 100%A where it contributed 8%) in the native stream, while *A. tetracladia* (11%–29%) and *T. elegans* (20%–33%) were more abundant in *C. sativa* litter, and *H. chaetoclada* (46%–82%) was more abundant in *A. melanoxylon*, in both streams (Table S7).

The aquatic hyphomycete community structure differed significantly between streams and among litter treatments (three-way multivariate PERMANOVA and PERMDISP analysis, $p \leq 0.008$), and there was a significant interaction between both factors ($p \leq 0.006$) (Figure 6; Table S9). Differences in aquatic hyphomycete community structure between streams in all litter treatments (pairwise tests, $p < 0.001$), occurred mainly due to the higher contribution of *A. filiformis* to conidial production in the native stream, and of *H. chaetoclada* and *D. foliicola* in the invaded stream (Table S7). Aquatic hyphomycete community structure, in both streams, did not significantly differ among 100%C, 75%C + 25%A and 50%C + 50%A litter

FIGURE 3 Fungal biomass on litter evenness treatments (100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A; C, *Castanea sativa*; A, *Acacia melanoxylon*) (a, d), and on C. *sativa* (b, e) and A. *melanoxylon* (c, f) litter from litter evenness treatments incubated in the native and in the invaded stream located in Serra da Lousã (central Portugal), over 77 days. Values are means \pm SE ($n = 3$)

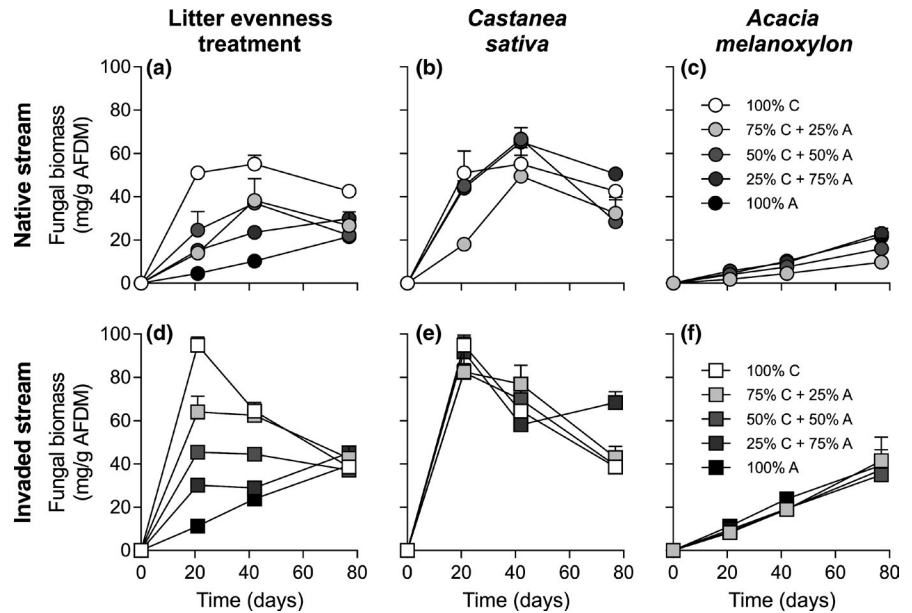
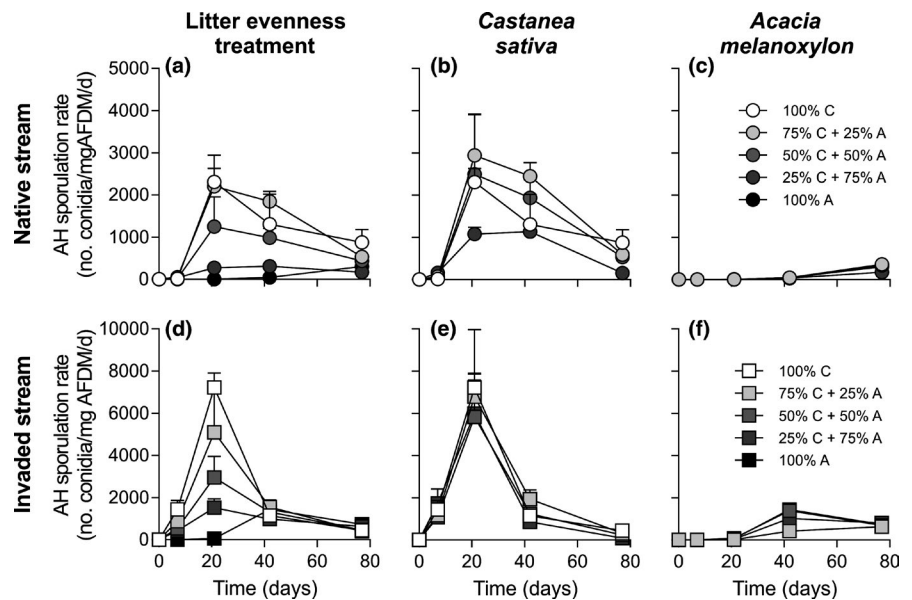


FIGURE 4 Aquatic hyphomycetes (AH) sporulation rate on litter evenness treatments (100%C, 75%C + 25%A, 50%C + 50%A, 25%C + 75%A and 100%A; C, *Castanea sativa*; A, *Acacia melanoxylon*) (a, d), and on C. *sativa* (b, e) and A. *melanoxylon* (c, f) litter from litter evenness treatments incubated in the native and in the invaded stream located in Serra da Lousã (central Portugal), over 77 days. Values are means \pm SE ($n = 3$)



treatments (pairwise tests, $p \geq 0.057$), but it differed between these and 25%C + 75%A and 100%A litter treatments ($p \leq 0.014$), with the exception of 50%C + 50%A and 25%C + 75%A litter treatments in the invaded stream that did not differ ($p = 0.080$), and differed between the 25%C + 75%A and the 100%A litter treatments, in both streams ($p < 0.001$) (Table S7; Figure 6). In the native stream, *H. chaetoclada*, *T. elegans*, *A. tetracladia*, *A. filiformis*, and *T. acuminatus* were the species that most contributed to the dissimilarities observed between 100%C, 75%C + 25%A and 50%C + 50%A litter treatments, and 25%C + 75%A and 100%A treatments (SIMPER analysis average dissimilarity $\geq 49\%$) (Table S10), while in the invaded stream it was *H. chaetoclada*, *T. elegans*, *D. foliicola*, and *A. tetracladia* (average dissimilarity $\geq 38\%$) (Table S11). Nevertheless, the aquatic hyphomycete community in the 100%A litter treatment was the one that differed most from the communities in the other litter treatments, in both

streams (SIMPER analysis average dissimilarity $\geq 80\%$ in the native and $\geq 59\%$ in the invaded stream; Tables S10 and S11, respectively). Additionally, the 100%A litter treatment had the lowest aquatic hyphomycete species richness (15 vs. 20–22 in other litter treatments in the native stream; 13 vs. 15–19 in other litter treatments in the invaded stream), and communities were clearly dominated by *H. chaetoclada* (76% and 82% in the native and the invaded stream, respectively), while in the other litter treatments conidia were more evenly distributed by *A. filiformis*, *A. tetracladia*, *D. foliicola*, and *T. elegans* ($\leq 29\%$) (Table S7). It also was interesting to note that in the invaded stream, litter mixing promoted aquatic hyphomycete species richness (18–19 species) compared with *C. sativa* and *A. melanoxylon* incubated alone (15 and 13 species, respectively), while in the native stream aquatic hyphomycete species richness was similar in litter mixtures and *C. sativa* litter alone (20–22 species) (Table S7).

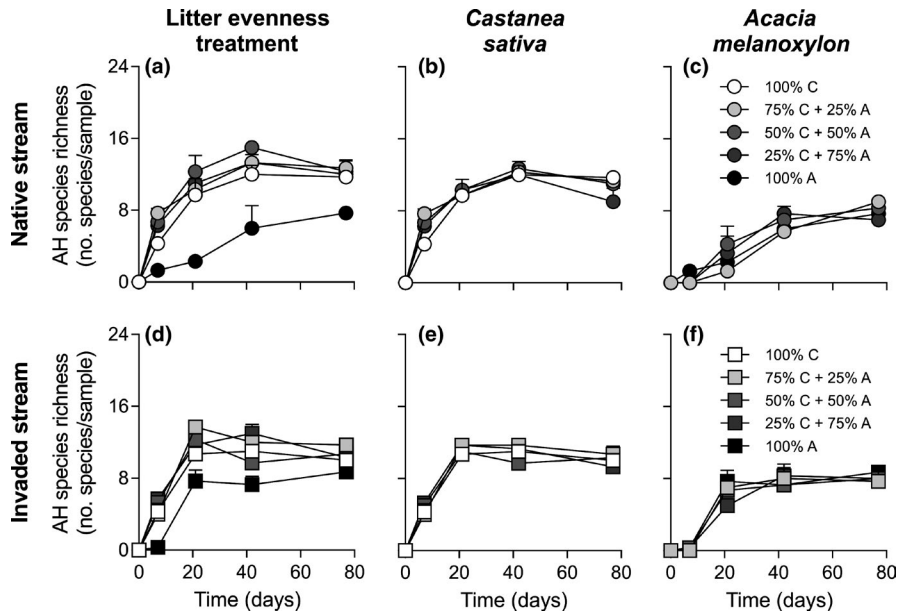


FIGURE 5 Aquatic hyphomycetes (AH) species richness on litter evenness treatments (100% C, 75% C + 25% A, 50% C + 50% A, 25% C + 75% A and 100% A; C, *Castanea sativa*; A, *Acacia melanoxylon*) (a, d), and on *C. sativa* (b, e) and *A. melanoxylon* (c, f) litter from litter evenness treatments incubated in the native and in the invaded stream located in Serra da Lousã (central Portugal), over 77 days. Values are means \pm SE ($n = 3$)

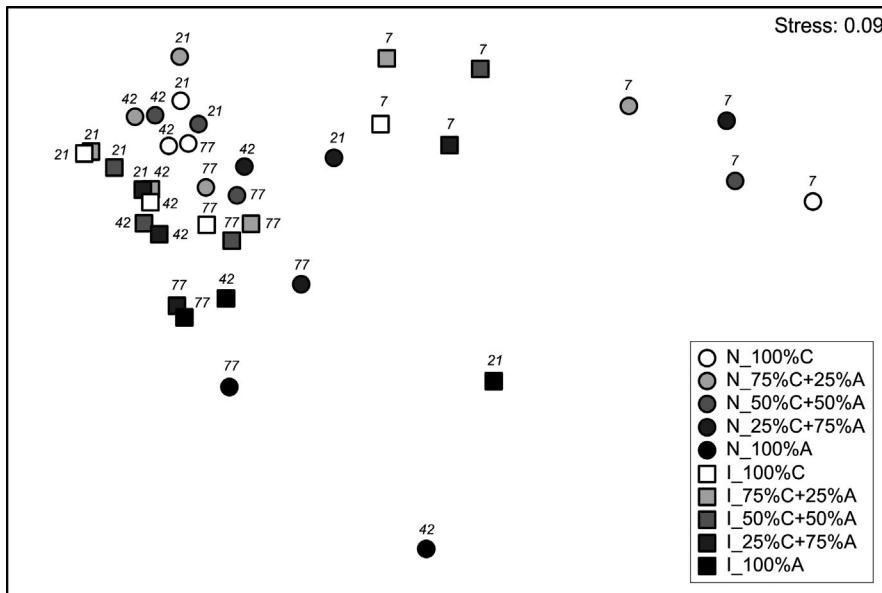


FIGURE 6 Ordination (nMDS) of aquatic hyphomycete communities (based on conidia production) associated with litter evenness treatments (100% C, 75% C + 25% A, 50% C + 50% A and 25% C + 75% A, 100% A; C, *Castanea sativa*; A, *Acacia melanoxylon*) in the native (N) and in the invaded (I) stream located in Serra da Lousã (central Portugal), after 7, 21, 42 and 77 days of stream immersion

4 | DISCUSSION

The invasion of native deciduous riparian forests by exotic N-fixing *Acacia* species is expected to impair small stream ecosystems, as a consequence of alterations in the diversity and quality of litter inputs from the riparian vegetation, and changes in water N concentration. In this study, litter decomposition rates, and microbial decomposer activity, species richness, and community structure were affected by litter diversity (species evenness) and quality, generally decreasing as the proportion of *A. melanoxylon* litter increased in litter treatments, probably because the native and the invasive litter species had very dissimilar characteristics. Litter diversity had small, but significant, nonadditive, antagonistic effects on litter decomposition in the native and the invaded stream. Additionally, litter decomposition rates and microbial decomposer activity were stimulated, and the

community structure was altered by the higher water N concentration in the invaded stream, probably promoted by the invasion of native deciduous riparian forests by N-fixing *Acacia* species.

4.1 | Litter decomposition rates and microbial decomposer activity decrease with increases in the proportion of *A. melanoxylon* litter

Litter decomposition rates and microbial decomposer activity decreased as the proportion of *A. melanoxylon* litter increased in litter evenness treatments, being 57%–62% lower for *A. melanoxylon* compared with *C. sativa* in 100% litter treatments. This suggests that nutrient cycling through the detrital food web will probably slow down as the proportion of *Acacia* species increases in invaded

riparian forests. Decreases of litter decomposition rates and microbial decomposer activity with increases in the proportion of *A. melanoxylon* litter were probably a result of the decreased P concentration and increased lignin concentration, and C:P and N:P ratios on litter treatments with the increasing proportion of *A. melanoxylon*. These results were expected since litter decomposition rates and microbial decomposer activity strongly depend on litter physical and chemical characteristics (e.g., Ferreira et al., 2012; Ferreira & Graça, 2016; Ferreira, Raposeiro, et al., 2016; Kominoski et al., 2009; Lecerf et al., 2007, 2011; Lima-Fernandes et al., 2015; Pereira & Ferreira, 2021; Rosemond et al., 2010). Indeed, litter that is soft and has low concentrations of refractory compounds, and high concentrations of nutrients is generally preferred by microbial decomposers, thus decomposing faster than recalcitrant litter (Gessner & Chauvet, 1994; Lecerf et al., 2007; Lima-Fernandes et al., 2015; Pereira & Ferreira, 2021; Pereira et al., 2016, 2021; Schindler & Gessner, 2009). In this study, *A. melanoxylon* litter was tougher, had lower SLA, higher lignin concentration, and higher C:P and N:P ratios than *C. sativa*, which probably limited microbial colonisation and activity. *Acacia melanoxylon* also had higher N concentration than *C. sativa*, but access to litter nutrients was probably hampered by the high lignin concentration. This was expected since the concentration of refractory compounds is often more important than litter nutrients in controlling microbial activity and litter decomposition (Frainer et al., 2015; Jabiol et al., 2019; Ramos et al., 2021; Schindler & Gessner, 2009). *Acacia melanoxylon* litter can thus be considered a very poor resource for aquatic microbial decomposers, as found previously in other studies (Campbell et al., 1992; Ferreira, Raposeiro, et al., 2016; O'Keefe & Lake, 1987; Pereira & Ferreira, 2021; Pereira et al., 2021; Raposeiro et al., 2014).

Despite differences in litter characteristics between species, it was also interesting to note that *C. sativa* decomposition rates were not affected by the presence of *A. melanoxylon*, and vice versa, as decomposition rates of individual litter species did not differ among litter treatments. On the contrary, aquatic hyphomycete sporulation rates in *A. melanoxylon* decreased with the increasing proportion of *C. sativa* in litter mixtures. Although in this study polyphenols concentration did not significantly differ between litter species, it was slightly higher in *C. sativa* than in *A. melanoxylon*. Thus, it is possible that polyphenol compounds could have inhibited microbial activity in *A. melanoxylon*, since they have been shown to have antimicrobial properties (Constabel et al., 2014; Martín et al., 2008). However, it is important to note that for practical reasons, *C. sativa* leaves were collected in a mountain range near to that where the study streams were located, even though *C. sativa* trees are also present in Serra da Lousã and in the riparian vegetation of the native stream. Since *C. sativa* leaves used in the experiment were not "local", it is possible that their decomposition was slower than if local leaves had been used, according with the "home-field advantage" hypothesis that postulates that decomposers are more adapted to local ("home") litter than litter from away. Still, the likelihood of results being affected by "home-field

advantage" is low since *C. sativa* leaves are of relatively high quality and there seems not to be strong support for the "home-field advantage" hypothesis in streams (Fenoy et al., 2016; Fugère et al., 2020; Yeung et al., 2019), where it may hold true only for litter that is challenging (i.e., low quality) and requires "specialized" decomposers (Yeung et al., 2019). Nonetheless, if, in this study, *C. sativa* litter decomposition was indeed slower than it would have been if local *C. sativa* leaves had been used, then the difference observed between *C. sativa* and *A. melanoxylon* was conservative and would have been larger if local leaves had been used.

4.2 | Small antagonistic effects of litter diversity on litter decomposition vary between streams

Litter diversity had small, but significant, antagonistic effects on litter decomposition in streams. Antagonistic diversity effects on litter decomposition most likely occurred because the native *C. sativa* and the invasive *A. melanoxylon* litter had very different physical and chemical characteristics (e.g., Kominoski et al., 2009; Lecerf et al., 2011; Swan et al., 2009). The transfer of polyphenolic leachates from *C. sativa* to *A. melanoxylon* litter probably inhibited the already reduced microbial activity on the latter species, thus slightly decreasing decomposition rates in the mixture. However, while in the native stream antagonistic effects occurred in litter treatments with even or higher proportions of *A. melanoxylon*, in the invaded stream they occurred in litter treatments with even or higher proportions of *C. sativa*. Differences in treatments showing antagonistic effects between streams suggest that diversity effects (species evenness) on litter decomposition may have been controlled by two non-exclusive conditions. On the one hand, diversity effects in litter treatments may have been sensitive to the dominant litter in the streambed. In each stream, there was no diversity effect for the litter treatment in which the dominant litter matched that of the streambed (i.e., 75%C + 25%A treatment in the native stream where *C. sativa* contributes most to the benthic organic matter standing stock and 25%C + 75%A treatment in the invaded stream where *Acacia* spp. contribute most to the benthic organic matter standing stock). As litter treatments were in contact with the streambed, and decomposers associated with the litter are under the general effect of litter diversity and quality of the surrounding environment, it is possible that the benthic litter moderated diversity effects, and only when the proportion of litter not present in the benthos increased in litter treatments diversity effects became evident. On the other hand, water N concentration could have modulated diversity effects on litter decomposition (Ferreira & Graça, 2016; Lima-Fernandes et al., 2015; Rosemond et al., 2010). Aquatic hyphomycetes can incorporate nutrients (e.g., N) from both the litter and the stream water (Gulis & Suberkropp, 2003; Suberkropp & Chauvet, 1995), and thus, higher water nutrient availability in the invaded stream may have made decomposers less dependent on litter nutrients.

4.3 | Litter decomposition rates and microbial decomposer activity are higher in the invaded than in the native stream

Litter decomposition rates and microbial decomposer activity were higher in the invaded than in the native stream, most likely because water N concentration was significantly higher in the former stream. Invasion of riparian forests by N-fixing species has been shown to increase water N concentration (Compton et al., 2003; Goldstein et al., 2009; Shaftel et al., 2012; Singh et al., 2019; Stewart et al., 2019; Wiegner et al., 2013), and the same trend has recently been observed in the case of the invasion of temperate deciduous riparian forests by *Acacia* species (Pereira et al., 2021). The increase in water N concentration in the invaded stream is most likely attributed to the invasion of the native forest by N-fixing *Acacia* species, because agricultural and industrial activity is almost non-existent and there are no other known N sources in the stream basin. In this stream, 94% of the riparian forest comprises *A. dealbata* (Pereira et al., 2021), and the supply of N to stream water may occur via: (a) direct input of *Acacia* litter into the stream and their decomposition in water; (b) leaching of N from the *Acacia* litter decomposing in the riparian *Acacia* stands through surface water runoff; and/or (c) flow of soluble N-rich root exudates from *Acacia* stands through ground water and hyporheic zones, as observed for other streams that flow through forests with high cover of N-fixing species (Compton et al., 2003; Goldstein et al., 2009; Shaftel et al., 2012; Singh et al., 2019; Stewart et al., 2019). Nevertheless, we should take into account that, even though water N concentration is still in the oligotrophic range in the invaded stream, probably it will increase in the future, as *Acacia* species become even more dominant in the stream basin, and the accumulation of organic material from *Acacia* trees in the riparian zone and the leaching of their soluble N compounds continues through time (Ferreira et al., 2021).

Small to moderate increases in water N concentration in oligotrophic streams generally stimulate microbial activity and litter decomposition (Ferreira et al., 2006; Gulis & Suberkropp, 2003; Rosemond et al., 2015). Indeed, it has been shown previously that even small increases in water N concentration promoted by *Acacia* species invasion can stimulate microbial decomposer activity and litter decomposition rates in N-limited streams (Pereira & Ferreira, 2021; Pereira et al., 2021). Nevertheless, it was interesting to note that the stimulation of litter decomposition by nutrients in stream water tended to be lower for litter treatments with higher proportions of *A. melanoxylon* litter (11%–24% stimulation) than for litter treatments with at least 50% *C. sativa* (13%–29% stimulation). Although increases in water nutrient concentrations generally have a stronger stimulatory effect in the decomposition of low-quality substrates, where microbial activity is nutrient-limited (e.g., Ferreira et al., 2006; Gulis et al., 2006; Gulis & Suberkropp, 2003; Pereira et al., 2016), the fact that *A. melanoxylon* litter had higher concentration of low-quality C (e.g., lignin) than *C. sativa* may have limited microbial use of dissolved nutrients (Jabiol et al., 2019).

Water temperature also was higher in the invaded than in the native stream. Therefore, the direct effect of temperature on litter decomposition rates were accounted for by expressing them on a degree-day basis. Decomposition rates also were corrected using the universal temperature dependence (Taddei et al., 2021); however, since statistical results were similar to those found when using decomposition rates on a degree-day basis, we opted to only show the results pertaining to the second, since this is the standard approach in decomposition experiments to correct for differences in temperature between streams. Still, temperature could have indirectly affected litter decomposition by intensifying nutrient effects. Indeed, it has been shown that increases in temperature may promote nutrient-enriched effects on microbial activity and litter decomposition (Fernandes et al., 2014; Ferreira & Chauvet, 2011). Nonetheless, in this study, the difference in water temperature between these temperate streams was small ($\sim 1^\circ\text{C}$), and probably did not have a measurable effect in microbial activity and litter decomposition.

4.4 | Aquatic hyphomycete community structure differs among litter evenness treatments and between streams

Aquatic hyphomycete communities differed among litter evenness treatments, being most dissimilar between treatments with even or higher proportion of *C. sativa* and those with higher proportion of *A. melanoxylon* litter. This was expected, since *A. melanoxylon* litter has been shown to be a poor-quality resource for aquatic hyphomycetes, probably owing to its high toughness that might hinder microbial colonisation and its high lignin concentration that might lead to C-limitation of microbial activity (Ferreira, Raposeiro, et al., 2016; Pereira & Ferreira, 2021; Pereira et al., 2021). Therefore, reductions in resource diversity promoted by *Acacia* species invasion, with the replacement of diverse deciduous forests by monospecific stands of *Acacia* trees, may lead to reductions in species richness and alterations in the community structure of aquatic microbial decomposers (Ferreira, Castela, et al., 2016; Laitung & Chauvet, 2005; Lecerf et al., 2005; Pereira & Ferreira, 2021; Pereira et al., 2021). Nevertheless, it was interesting to note that the community composition of aquatic hyphomycetes in 75%C + 25%A and 50%C + 50%A litter treatments did not differ from that present when *C. sativa* was incubated alone, despite the increasing proportion of *A. melanoxylon* in mixtures. This suggests that the maintenance of native riparian forests that contribute with high-quality litter to streams flowing through forests invaded by *Acacia* species might partially buffer the negative effects of the invasion on decomposer communities. However, the observed effects of litter species evenness in the community structure of aquatic microbial decomposers probably cannot be generalised for all invasive *Acacia* species because these differ in their litter characteristics. For instance, a previous experiment has shown that the aquatic hyphomycete community present in *A. dealbata* litter was very similar to that found in native *A. glutinosa* and *Q. robur* (Pereira & Ferreira, 2021).

Differences in aquatic hyphomycete species richness and community structure were also observed between streams. Aquatic hyphomycete species richness was higher in the native than in the invaded stream, probably because the former flows through a diverse native forest of deciduous trees, while the latter flows through a nearly monospecific stand of *Acacia* trees. Indeed, previous studies have found high diversity of aquatic microbial decomposers in streams flowing through more than less diverse forests (Bärlocher & Graça, 2002; Ferreira, Castela, et al., 2016; Laitung & Chauvet, 2005; Lecerf et al., 2005; Pereira & Ferreira, 2021; Pereira et al., 2021). For instance, *A. filiformis* was one of the species that most contributed to the community in the native stream but had an extremely low contribution in the invaded stream, as observed recently (Pereira et al., 2021). Nevertheless, it was interesting to note that when considering individual litter species, aquatic hyphomycete species richness per sampling date in *C. sativa* and *A. melanoxydon* litter differed significantly between streams. In *C. sativa* litter, aquatic hyphomycete species richness was higher in the native stream, while in *A. melanoxydon*, species richness was higher in the invaded stream. It seems that in the invaded stream, the small increase in water N concentration not only stimulated the activity of microbial decomposers in *A. melanoxydon*, but also favoured the colonisation of this recalcitrant resource by a richer community of aquatic hyphomycete species.

Differences in the aquatic hyphomycete community structure between streams probably occurred more as a result of niche-driven effects (e.g., environmental filtering) than neutral effects (i.e., stochastic processes). Although trophically similar, aquatic hyphomycete species can differ in multiple aspects that may determine their (reproductive) success under changing environmental conditions (e.g., different conidia size, stoichiometric requirements, enzymatic capacity) (Abdel-Raheem & Ali, 2004; Brosted et al., 2017; Gessner & Chauvet, 1994; Zemek et al., 1985). Indeed, previous studies have shown changes in aquatic hyphomycete community structure (from a common species pool) with nutrient enrichment (Artigas et al., 2008; Ferreira & Graça, 2016; Gulis & Suberkropp, 2003). A compilation of the aquatic hyphomycete species (based on conidial production) identified in this and two previous experiments (Pereira & Ferreira, 2021; Pereira et al., 2021) performed in the same streams showed that aquatic hyphomycete communities had a very similar species pool: from a total of 25 species identified across the three studies, 22 were found in both streams, whereas two species were only found in the native stream and one species was only found in the invaded stream; in addition, the three species that were unique to one stream are generally rare species (data not shown). Therefore, the environmental conditions of each stream (e.g., nutrient concentrations in water, the diversity of tree species in the riparian vegetation) may have affected the reproductive activity of aquatic hyphomycetes, and since results on the community structure were based on the sporulation rate of each species, their structure differed significantly between streams.

5 | CONCLUSION

Our results suggest that increases in the proportion of recalcitrant *Acacia* litter in streams flowing through invaded deciduous riparian forests will slow down litter decomposition, decrease microbial decomposer activity and alter their community structure. Additionally, increases in water N concentration resulting from invasion by N-fixing *Acacia* species will accelerate litter decomposition, and stimulate the activity of associated microbial decomposers. Nevertheless, the stimulatory effects of increases in water N concentration, on microbial activity and litter decomposition probably will not counteract the inhibitory effects promoted by increases in litter recalcitrance. Thus, litter decomposition potential, and consequently nutrient and energy transfer to higher trophic levels, will be lower in invaded than in native streams. However, the preservation of native deciduous trees contributing with high-quality litter to streams may help mitigating direct effects of inputs of recalcitrant *Acacia* litter. Nonetheless, effects of *Acacia* species invasion may vary among *Acacia* species that may show different litter characteristics.

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AUTHOR CONTRIBUTIONS

Both authors contributed to the study conception and design. Material preparation, deployment in the field, sampling and laboratory processing were done by Ana Pereira and Verónica Ferreira. Determinations of litter AFDM, fungal biomass and identification of aquatic hyphomycetes were done by Ana Pereira. The statistical analysis and the first draft of the manuscript was written by Ana Pereira, and Verónica Ferreira commented and contributed to all versions of the manuscript. Both authors read and approved the manuscript final version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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