Key plant species and detritivores drive diversity effects on instream leaf litter decomposition more than functional diversity: A microcosm study

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HIGHLIGHTS

- Effects of functional diversity loss on instream litter decomposition were assessed.
- Diversity effects were mostly controlled by the N-fixing *Alnus glutinosa*.
- Overall positive diversity effects were detected for detritivore-mediated processes.
- Overall negative diversity effects were detected for microbially-mediated processes.
- Key plant species and detritivores, more than functional diversity, were the essential drivers of diversity effects.

GRAPHICAL ABSTRACT
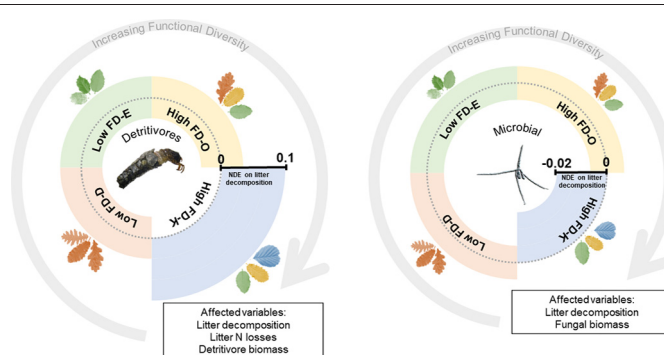
ABSTRACT

Anthropogenic impacts on freshwater ecosystems cause critical losses of biodiversity that can in turn impair key processes such as decomposition and nutrient cycling. Forest streams are mainly subsidized by terrestrial organic detritus, so their functioning and conservation status can be altered by changes in forest biodiversity and composition, particularly if these changes involve the replacement of functional groups or the loss of key species. We examined this issue using a microcosm experiment where we manipulated plant functional diversity (FD) (monocultures and low-FD and high-FD mixtures, resulting from different combinations of deciduous and evergreen *Quercus* species) and the presence of a key species (*Alnus glutinosa*), all in presence and absence of detritivores, and assessed effects on litter decomposition, nutrient cycling, and fungal and detritivore biomass. We found (i) positive diversity effects on detritivore-mediated decomposition, litter nutrient losses and detritivore biomass exclusively when *A. glutinosa* was present; and (ii) negative effects on the same processes when microbially mediated and on fungal biomass. Most positive trends could be explained by the higher litter palatability and litter trait variability obtained with the inclusion of alder leaves in the mixture. Our results support the hypothesis of a consistent slowing down of the decomposition process as a result of plant biodiversity loss, and hence effects on stream ecosystem functioning, especially when a key (N-fixing) species is lost; and underscore the importance of detritivores as drivers of plant diversity effects in the studied ecosystem processes.

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1. Introduction

The current human-induced biodiversity decline (Ceballos and Ehrlich, 2018) and its consequences for ecosystem functioning (hereafter B-EF) have attracted much attention since the seminal paper by Naeem et al. (1994). Headwater stream ecosystems in forested regions may be especially sensitive to plant biodiversity loss, owing to their dependence on allochthonous organic matter, mostly in the form of leaf litter (Kominoski et al., 2011; Wallace et al., 1997). Instream litter decomposition is a crucial process involving the cycling of nutrients and the production of microbial and invertebrate biomass (Marks, 2019). Therefore, ongoing changes in riparian vegetation affecting litter inputs (e.g. dominance of evergreen versus deciduous plants; Kominoski et al., 2013; Salinas et al., 2018) may alter the stream food web structure (Bärlocher and Graça, 2002; Martínez et al., 2013; Seena et al., 2017) as well as biogeochemical cycles at multiple scales (Battin et al., 2009; Casas et al., 2013).

Plant biodiversity loss effects on instream decomposition have been addressed by numerous studies. However, their outcomes have been idiosyncratic (Gessner et al., 2010), showing positive (e.g., Gartner and Cardon, 2004; Leroy and Marks, 2006) to negative diversity effects (e.g., Swan and Palmer, 2006). These contrasting results may arise from differences in experimental designs (field vs. laboratory experiments), the selected plant species or the use of different diversity measures. An alternative approach to species richness is to focus on functional diversity (FD), i.e. the diversity of functional traits, which has been reported to have a pivotal role on ecosystem processes (Petchey and Gaston, 2006) by favoring different organisms (niche or resource partitioning; Finke and Snyder, 2008). However, there is no consensus about which is the most suitable FD measure (Flynn et al., 2011; Petchey et al., 2009), or which traits are the most relevant for a particular process.

Another controversial question relates to the primary role of single species, in particular key species (also called keystone process species sensu Folke et al., 1996), which might drive critical processes and thus determine the overall ecosystem functioning (Ellison et al., 2005). Litter from these key species could promote diversity effects through a variety of underlying mechanisms, including an increase in the mean value of the nutrient pool in litter mixtures (mass ratio hypothesis; Grime, 1998), or via nutrient transfer between litter types (Gessner et al., 2010; Tiunov, 2009).

Our study addresses two main questions. First, by mixing tree species belonging to the same or different functional types in terms of their leaf habits (deciduous, semi-deciduous or evergreen), we explored plant FD effects on several stream ecosystem processes (litter decomposition, nutrient cycling and changes in biomass of fungi and detritivores) and the relative role of microbial decomposers and detritivores as drivers of these effects. Second, we examined the role of black alder, *Alnus glutinosa* (L.) Gaertn., a deciduous nitrogen (N)-fixing plant with soft, nutrient-rich and fast decomposing litter (Casas et al., 2013; Hladyz et al., 2009), as key species with direct repercussions on the above processes (e.g., Graça et al., 2015; Pérez et al., 2021; Piccolo and Wipfli, 2002) and B-EF relationships (Alonso et al., 2021; López-Rojo et al., 2018). In Europe, populations of *A. glutinosa* are declining since 1990 (Bjelke et al., 2016; Gibbs et al., 1999) due to a fungal disease caused by *Phytophthora alni* "sensu lato" (Husson et al., 2015). We used microcosms, with and without a stream detritivore to conduct an experiment using monocultures, low-FD and high-FD mixtures (resulting from different combinations of deciduous and evergreen *Quercus* species) and the key species *A. glutinosa*. We tested the following hypotheses: (1) FD increases trait variability of litter mixtures (Flynn et al., 2011; Hillebrand and Matthiessen, 2009); (2) litter FD enhances decomposition and nutrient cycling through resource partitioning (Handa et al., 2014; López-Rojo et al., 2019) and (3) leads to a higher fungal (Kominoski et al., 2009) and detritivore biomass through a balanced diet effect (DeMott, 1998; Duffy et al., 2007);

(4) the above diversity effects are greater when a key plant species is present due to overall increased nutrient content and/or increased trait variability (Larrañaga et al., 2020; López-Rojo et al., 2018); and (5) such effects are mostly mediated by detritivores (Tonin et al., 2017).

2. Material and methods

2.1. Selection of litter and detritivore species

We selected 7 oak species belonging to 3 different plant functional groups in terms of their leaf habits, and consequently with regard to leaf traits and carbon (C) gain strategies (see Escudero et al., 2017; Gil-Pelegrín et al., 2017 for more details) to examine effects of plant FD on stream processes: 3 deciduous (*Quercus robur* L., *Q. canariensis* Willd. and *Q. pyrenaica* Willd.), 1 semi-deciduous (*Q. faginea* Lam.) and 3 evergreen species (*Q. coccifera* L., *Q. suber* L. and *Q. rotundifolia* Lam.). Deciduous *Quercus* species have leaves that show functional traits values such as greater leaf area, and lower Leaf Mass per Area (LMA) and leaf thickness (considered proxies for mass allocation and related processes, Onoda et al., 2011), and shorter leaf lifespan than leaves of evergreen *Quercus* species (Escudero et al., 2017). *Quercus faginea* is a semi-deciduous tree (Sanz-Pérez et al., 2009), i.e. its old and withered leaves remain on the plant for the most of the winter until the emergence of new leaves the following spring (see Veselá et al., 2018). Additionally, we chose the deciduous N-fixing *A. glutinosa* as key species (Pérez et al., 2021). The 8 species covered a broad litter quality spectrum (Tables 1, S1). Leaves were collected in autumn 2017 from forests of different catchments in Southern Spain, except for *Q. robur*, which was collected in Northern Spain as it is absent at lower latitudes (Table S2). Deciduous leaves were gathered from the forest floor just after senescence and evergreen leaves were collected from dry, broken branches, with no symptoms of diseases. Leaves were air dried at room temperature (20–23 °C) for 1 week and stored in darkness until used for experimentation.

We selected larvae of the caddisfly *Allogamus mortoni* Navás (hereafter *Allogamus* or detritivores), an endemic leaf-shredding species common in streams of the Iberian Peninsula (Ruiz-García et al., 2004). This species is widespread within the Iberian Peninsula and likely coexist with all the plant species selected. Detritivores were collected from Río del Pueblo, a second-order stream located at 1405 m a.s.l in the Sierra Nevada Natural Park (37.15°N, 3.15°W; Southeast Spain). Riparian vegetation consists of mixed deciduous tree species dominated by alder groves (*Alnus glutinosa*) and plantations of *Populus nigra* L., followed out to the slopes by natural forests of the evergreen holm-oak (*Quercus rotundifolia*) and plantations of the conifer *Pinus pinaster* Aiton. Other frequent plant species encountered in the stream bank include *Salix atrocinerea* Brot., *Castanea sativa* Mill, *Populus alba* L., or *Rubus ulmifolius* Schott. Detritivores were acclimated to experimental conditions (see below) for 1 week and starved for 48 h just prior to starting the experiment. Initial detritivore dry mass (DM) was estimated from a case aperture diameter (CD) (mm) / DM (mg) relationship, using additional *Allogamus* larvae (DM = 0.097 e$^{1.362 \, CD}$, R^2 = 0.87, $p < 0.001$, root mean square error = 1.64, $n = 55$). CD was measured under a binocular microscope (0.1 mm), and DM was weighed (0.1 mg) in freeze-dried uncased individuals that were later used to analyse their initial body N and phosphorus (P) (see Supplementary Methods in Supplementary Data).

2.2. Experimental setup

A total of 120 microcosms were placed in a temperature-controlled room set at 10 °C (the mean of hourly records obtained in the stream during the experiment season (April–May) in previous years) and a light:dark 12:12 h photoperiod (based on the natural cycle at that time of the year), and provided with continuous air supply (Fig. S1). Each microcosm consisted of a 500-mL glass jar containing 30 cm^3 of

Table 1
Experimental treatments of varying functional diversity, *Quercus* and *Alnus* species used in the microcosm experiment. Leaf litter quality index (LQI) of each species is showed between parenthesis and LQI and Rao's quadratic diversity (Rao's Q) of litter mixtures traits is presented in the corresponding columns.

Treat ID	Functional diversity	Functional group	Sp1	Sp2	Sp3	LQI	Rao's Q
Low FD-D	Low	Deciduous	*Q. robur* (0.36)	*Q. canariensis* (0.34)	*Q. pyrenaica* (0.35)	0.35	4.78
Low FD-E	Low	Evergreen	*Q. coccifera* (0.23)	*Q. suber* (0.35)	*Q. rotundifolia* (0.36)	0.31	4.86
High FD-O	High	Deciduous, semi-deciduous and evergreen	*Q. robur* (0.36)	*Q. faginea* (0.37)	*Q. rotundifolia* (0.36)	0.36	9.37
High FD-K	High	Deciduous and N-fixing, semi-deciduous and evergreen	*A. glutinosa* (0.58)	*Q. faginea* (0.37)	*Q. rotundifolia* (0.36)	0.43	10.59

stream sediment, previously furnaced (500 °C, 5 h) and washed with distilled water, and 400 mL of filtered (100 μm) stream water, which was oligotrophic (soluble reactive P: 7 μg P L^{-1}; nitrate-N: 24 μg N L^{-1}) and soft circumneutral (pH: 7.10; electrical conductivity: 60 μS cm^{-1}; alkalinity: 0.32 meq L^{-1}). Sediment and water were collected from the same stream as detritivores (Río del Pueblo). Just before the experiment, litter was cut ($\approx 2 \times 2$ cm, avoiding the basal midrib) and weighed to the nearest 0.1 mg. Each microcosm received 1.516 ± 0.001 g (mean $\pm$ SE) of air-dried litter pieces belonging to 1 (monocultures) or 3 plant species (litter mixtures containing 0.5 g per species), combined as shown in Table 1 to create the highest gradient of FD possible among all the potential combinations (Table S3) (12 treatments in total: 8 monocultures and 4 mixtures). Mixtures consisted of 2 low FD (deciduous: Low FD-D, or evergreen: Low FD-E, oak species) and 2 high FD treatments (one including all the leaf habits —deciduous, semi-deciduous and evergreen— within oak species: High FD-O, and the other including also the key species: High FD-K).

Litter fragments of the same species were color-coded and kept together using 3 safety pins per microcosm; the same was done in monocultures to mirror the conditions of mixtures. In each litter treatment, detritivores were added to 7 microcosms (2 larvae of *Allogamus* per microcosm); the other 3 remained without detritivores in order to separate their contribution from that of microorganisms (Fig. S1). Detritivore initial biomass per microcosm was on average 12.94 ± 0.98 mg (mean $\pm$ SE), without significant differences across litter treatments (One-way ANOVA, $p = 0.97$). The experiment lasted 42 days (April–May 2018). Larval mortality was recorded daily, and dead larvae (52 out of 244) were replaced by a new one only when both individuals died in a given jar (4 out of 84 microcosms with detritivores).

2.3. Experimental procedure

Before adding the detritivores to microcosms, litter fragments were incubated for 5 days in microcosms with aeration (with stream water renewal on the third day) to allow the leaching of soluble compounds and initial microbial colonization. Twenty-four extra microcosms without detritivores (3 per plant species in monoculture), containing ca. 1.5 g of litter, were used to measure initial (post-leaching; Fig. S2) ash-free dry mass (AFDM) and litter traits (Supplementary Methods). Thereafter, water was renewed weekly with filtered (100 μm) stream water collected the same day. On the last day, litter material was separated by species, and 5 discs (12 mm ø) of each species (3 replicates with and 3 without detritivores per treatment) were cut out with a cork borer, freeze-dried, weighed and used to estimate fungal biomass, which was measured through ergosterol concentration following Suberkropp and Weyers (1996) with some modifications (Supplementary Methods). The remaining litter material was oven-dried (70 °C, 72 h), weighed to determine final DM, and ground to leaf powder (Mixer Mill RETSCH MM 200). An aliquot of each sample was furnaced (550 °C, 5 h) and weighed to estimate final AFDM, and another aliquot was used for elemental analyses (C, N and P; Supplementary Methods). At the end of the experiment, detritivores from each microcosm were

starved for 48 h. Then, the diameter of their cases was measured as above and individuals were uncased, freeze-dried and stored until N and P analyses (Supplementary Methods).

2.4. Data analyses

We examined trait variability among litter treatments using principal component analysis (PCA; *prcomp* function in the 'stats' package of R statistical software (R Core Team, 2020)). Previously, a Pearson correlation analysis was performed to remove litter traits with high (>0.85) collinearity (Fig. S3). Twelve litter traits were included in the PCA: toughness, N, P, N:P molar ratio, hemicellulose, lignin, total phenolics, Ca, K, Mg, Si and non-structural carbohydrates (NSC). To test our 1st hypothesis we assessed trait variability in litter mixtures using the Rao's quadratic diversity coefficient (Rao, 1982), widely applied in functional ecology analyses [Rao's Q; *dbFD* function in the 'FD' R package (Laliberté et al., 2014)]. Statistical differences among species or mixture traits were assessed using one-way ANOVA and Tukey multiple comparisons tests with the *anova* and *TukeyHSD* functions of 'stats' R package (R Core Team, 2020). *Log* or arcsin transformations of variables were used when required.

Litter palatability was estimated using the litter quality index (LQI) equation proposed by Solagaistua et al. (2019): LQI: $1 - [(T_1 / (n \times T_{1(max)})) + ... + (T_n / (n \times T_{n(max)}))]$; where T is the average of the measured value for the trait and n the number of measured traits. In order to ease interpretation, we selected a limited number of traits (5 out of 20: toughness, C:N and C:P molar ratios, phenols and NSC) that are relevant for detritivore- (e.g., Hladyz et al., 2009) and microbially-mediated decomposition (Fernandes et al., 2012; López-Rojo et al., 2020), being the latter usually favored by a high nutrient and structural carbohydrates content.

Litter decomposition was quantified as the proportion of litter mass loss [LML = (initial − final AFDM) / (initial AFDM)], after correcting initial litter mass for humidity, ash content and leaching losses. LML in microcosms without detritivores (i.e., microbially-mediated LML) was used to correct initial AFDM in microcosms with detritivores and thus isolate detritivore-mediated LML, allowing us to partially test our 5th hypothesis (i.e. FD effects are mostly mediated by detritivores). Litter N loss (LNL) and litter P loss (LPL) were calculated as: (initial − final) / initial N or P content. Positive values represented nutrient mineralization, and negative ones nutrient immobilization. Detritivore relative growth (DMG), detritivore N gain (DNG) and detritivore P gain (DPG) were quantified as the proportional change in detritivore DM, N and P content, respectively [(final − initial) / initial DM, N or P content]; and were estimated only in those microcosms with no animal replacements. Finally, proportional changes in nutrient concentration of litter and detritivores were determined as: [(final − initial) / (initial N or P %)].

We examined differences in all response variables (LML, LNL, LPL, ergosterol, DMG, DNG and DPG) among monocultures and among litter mixtures using one-way ANOVAs and Tukey multiple comparisons tests (see above function and package). To avoid very complex models

with many interactions, we examined detritivore- and microbially-mediated variables separately. We applied the Kaplan-Meier test to compare *Allogamus* survivorship among treatments using the *survfit* function of 'survival' R package (Therneau, 2015). Any possible effect of mortality on other detritivore related variables was prevented standardizing by the number of days that each animal survived relative to the experimental time.

We explored our 2nd and 3rd hypotheses through the difference between observed (Obs.) and expected (Exp.) values (i.e. the net diversity effect, NDE = Obs. − Exp.), with expected values estimated based on monocultures. NDE was estimated in microcosms with and without detritivores enabling us to better assess our fifth hypothesis. To assess the magnitude of these effects we calculated the relationship between the NDE and expected values [NDE magnitude (%): (Obs. − Exp.) / Exp. × 100]. We explored differences among treatments with nonparametric bootstrapped 95% confidence intervals using the BCa method in the *boot.ci* function of 'boot' R package (Canty and Ripley, 2019), which allowed us to test if FD effects were greater when the key plant species is present (our 4th hypothesis). For LML and ergosterol, the NDE was partitioned into complementarity and selection effects using the additive partitioning method (Loreau and Hector, 2001). In the case of LML, we were able to split diversity effects for total, detritivore- and microbially-mediated decomposition, to further assess the role of detritivores on BE-F relationships (5th hypothesis). Complementarity effects are explained by synergistic (positive) or antagonistic (negative) interactions; and selection effects represent positive (or negative) effects due to the presence of a dominant species in the mixture, which enhances (or inhibits) overall decomposition of the mixture (Handa et al., 2014). In addition, we carried out intraspecific comparisons, i.e. we compared LML for each species in monocultures and in each mixture where present. We finally explored the relationships between litter palatability (LQI) or litter traits variability (Rao's Q) with the measured variables and diversity effects in mixtures using linear models (*lm* function of 'stats' R package) in order to better explain the observed patterns and separate the FD effects from that caused by the presence of the key species.

3. Results

3.1. Litter trait variation across species and mixtures

Trait variability within a mixture (represented by polygon size, Fig. 1) was the highest in High FD-K followed by High FD-O, Low FD-E and Low FD-D, matching the FD assessment by means of Rao's Q (Table 1). The first 2 dimensions of the PCA explained 68.7% of trait variation (Fig. 1, Table S1) and showed a clear separation of evergreen and deciduous species and their mixtures; however, low-FD and high-FD mixtures (which shared species) overlapped. PC1 mostly represented a gradient of structural and secondary metabolites, and PC2 represented a gradient in elemental composition. *A. glutinosa* was clearly separated from oak species due to its particularly high concentration of lignin and N. LQI (Table 1) was lowest for *Q. coccifera* and the evergreen mixture (Low FD-E), and highest for *A. glutinosa* and the High FD-K mixture. The third dimension of PCA explained 15.9% of traits variation and helped to further separate *A. glutinosa* from the rest of species.

3.2. Litter decomposition

In monocultures, the highest decomposition was found for *A. glutinosa* in presence of detritivores, and also for the semi-deciduous *Q. faginea* in their absence; decomposition was lowest for the deciduous *Q. robur* and *Q. canariensis* with and without detritivores (Fig. S4A, B). Decomposition of monocultures was explained by LQI only when detritivores where present ($R = 0.60$, $p < 0.001$); microbially-mediated decomposition showed a similar but not significant trend ($R = 0.37$, $p = 0.076$). The mixture with *A. glutinosa* (High FD-K) showed
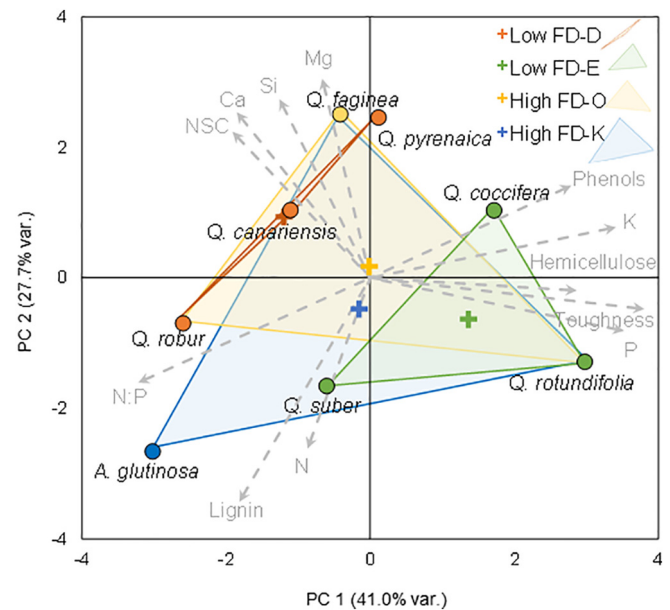
Fig. 1. Projection of the 2 first principal component axis showing differences among litter traits of the 8 individual species and 4 litter mixtures (Explained variance = 68.7%). Vectors represent each leaf trait weight and circles each plant litter position: deciduous in brown, semi-deciduous in yellow, evergreen in green and key species in blue. Triangular shapes represent the mixtures and crosses the weighted means of trait values calculated for each mixture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the highest decomposition and the deciduous mixture (Low FD-D) the lowest, regardless of detritivore presence (Fig. S4A, B), but only in their presence the difference was significant (One-way ANOVA; $F_{3,24} = 11.474$, $p < 0.001$). Decomposition of mixtures was positively related with Rao's Q, but LQI only explained decomposition in microcosms with detritivores (Table S4).

Net diversity effects (NDE) on decomposition were only significant in the High FD-K mixture, being positive for total (detritivores + microorganisms; Fig. 2A) and detritivore-mediated decomposition (Fig. 2B), but negative for microbially-mediated decomposition (Fig. 2C). The magnitude of these effects was 23.8%, 50.5% and 11.4% for total, detritivore-mediated and for microbial decomposition, respectively. When partitioning NDE (Fig. 2D–I), we found negative complementarity effects only in microbially-mediated decomposition of the High FD-K mixture. This mixture always showed positive selection effects. The NDE on detritivore-mediated decomposition was correlated with LQI, while selection effects on total and detritivore-mediated decomposition were correlated with both, LQI and Rao's Q (Table S4).

Furthermore, intraspecific comparisons of litter decomposition (i.e. comparison of LML of a particular species in monocultures and mixtures) highlighted significant mixing effects for some litter species (Fig. 3), being noteworthy the consistent pattern found for *A. glutinosa*: faster decomposition in the mixture (High FD-K) than in monocultures with detritivores, but lower without them.

3.3. Litter nutrient dynamics

When detritivores were present, LNL was mostly positive in monocultures and mixtures (Fig. S4C), accompanied, in general, by an increase of litter N concentration (N%; range of increase: 1.4–23.8%). LNL increased with LQI in monocultures ($R = 0.54$, $p = 0.007$) and also with Rao's Q in mixtures (Table S4). Mean NDE on LNL was positive for all mixtures, but only significant (24% increase) in the High FD-K mixture (Fig. 4A).

Without detritivores we observed net N immobilization (i.e., negative LNL) in low-diversity mixtures (Low FD-D and Low FD-
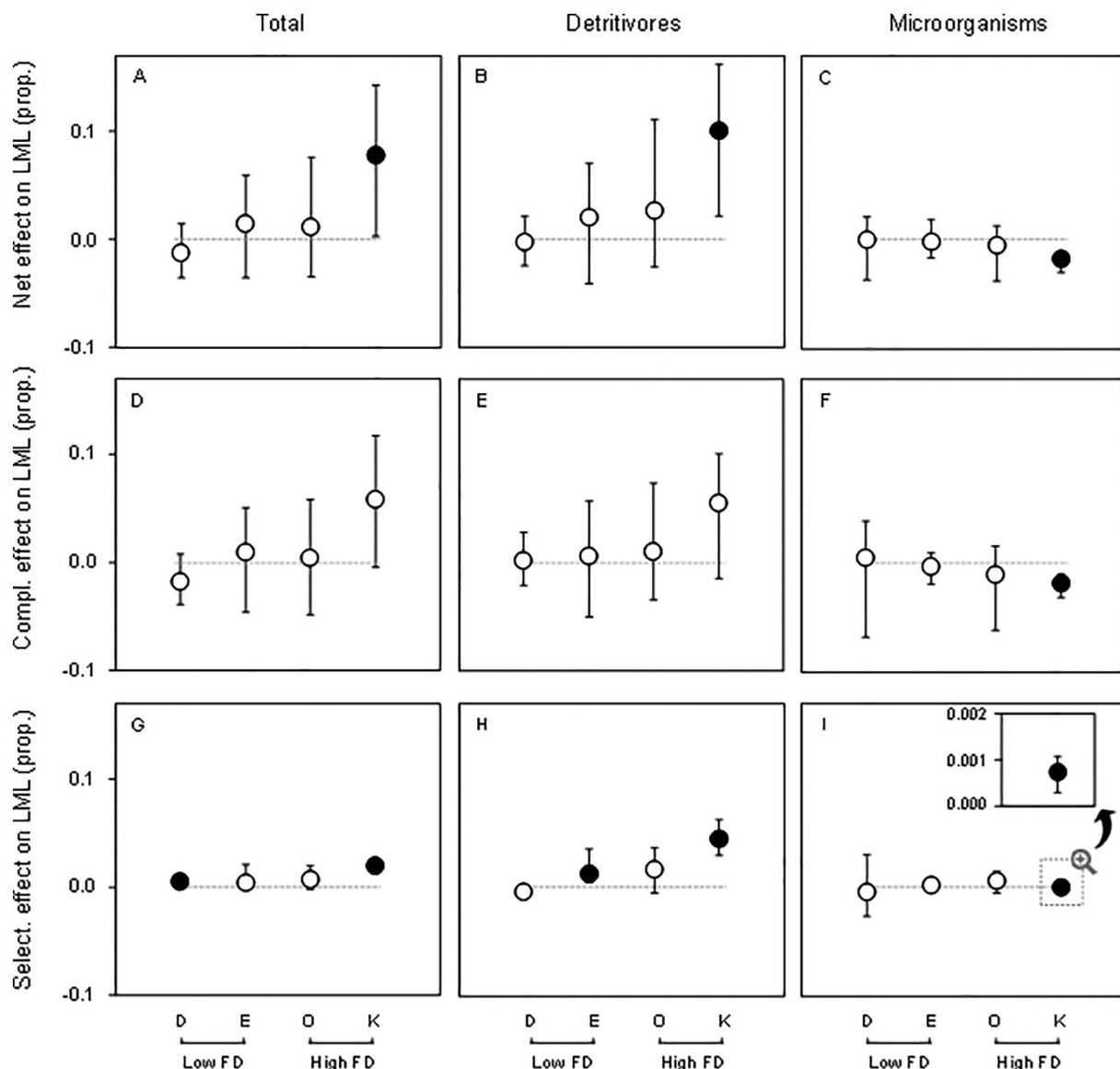
Fig. 2. Net diversity, complementarity and selection effects on total (A, D and G), detritivore-mediated (B, E and H) and microbially-mediated (C, F and I) litter mass loss (LML; proportion). Mean values (circles) and upper and lower limits of 95% nonparametric bootstrapped confidence intervals (whiskers) are presented. Dashed lines denote no-effect, i.e. the null expectation that mixtures do not differ from expected ones, estimated from monocultures. Closed circles represent intervals that reject the null hypothesis (i.e. confidence interval do not contain the 0-value) and open circles represent intervals that accept the null hypothesis.

E; Fig. S4D). In contrast, most monocultures and high-diversity mixtures (High FD-O and High FD-K; Fig. S4D) showed positive LNL. Litter N concentration (N%) generally increased (3.9–17.7%). LNL in mixtures was positively related with LQI and Rao's Q (Table S4). NDE on microbially-mediated LNL was negative in all mixtures (Fig. 4B).

In presence of detritivores, LPL was positive in mixtures and most monocultures (Fig. S4E), increasing with mixture's Rao's Q (Table S4). In these microcosms, litter P concentration (P%) generally increased in deciduous oaks (1.6–36.2%) but decreased in the other species. No diversity effects on LPL were detected in presence of detritivores (Fig. 4C).

Without detritivores, litter P content tended to increase (negative LPL values) in low-diversity mixtures (Low FD-D and Low FD-E), and to decrease (positive LPL values) in high-diversity mixtures (High FD-O and High FD-K; Fig. S4F), being positively related to Rao's Q (Table S4). Litter P concentration (P%) increased in all litter species (2.8–56.3%) except *Q. faginea* (11.3–14.4% of reduction). NDE was negative for all mixtures without detritivores, excepting High FD-O (Fig. 4D).

3.4. Fungal biomass

In monocultures, ergosterol concentration varied among species (with detritivores: $F_{7, 16} = 6.28$, $p = 0.001$; without detritivores: $F_{7, 16} = 3.01$, $p = 0.032$), being on average higher in deciduous than evergreen species (Fig. S4G, H). Similarly, comparisons across litter mixtures showed significant differences in ergosterol concentration within microcosms with ($F_{3, 8} = 10.79$, $p = 0.003$) and without ($F_{3, 8} = 12.68$, $p = 0.002$) detritivores, being the lowest in the evergreen mixture (Fig. S4G, H). NDE on fungal biomass was always negative in microcosms with detritivores (effects from −13.5% to −21.4%), and more variable without them (Fig. 5A, B), being highest (ca. −35%) in Low FD-E and High FD-K mixtures (Fig. S4H). Complementarity effects followed the net diversity effects' pattern (Fig. 5C, D). Selection effects, always of lower magnitude than complementarity, arose only in few combinations (Fig. 5E, F). No relationships between ergosterol accrual and LQI or Rao's Q were found (Table S4).
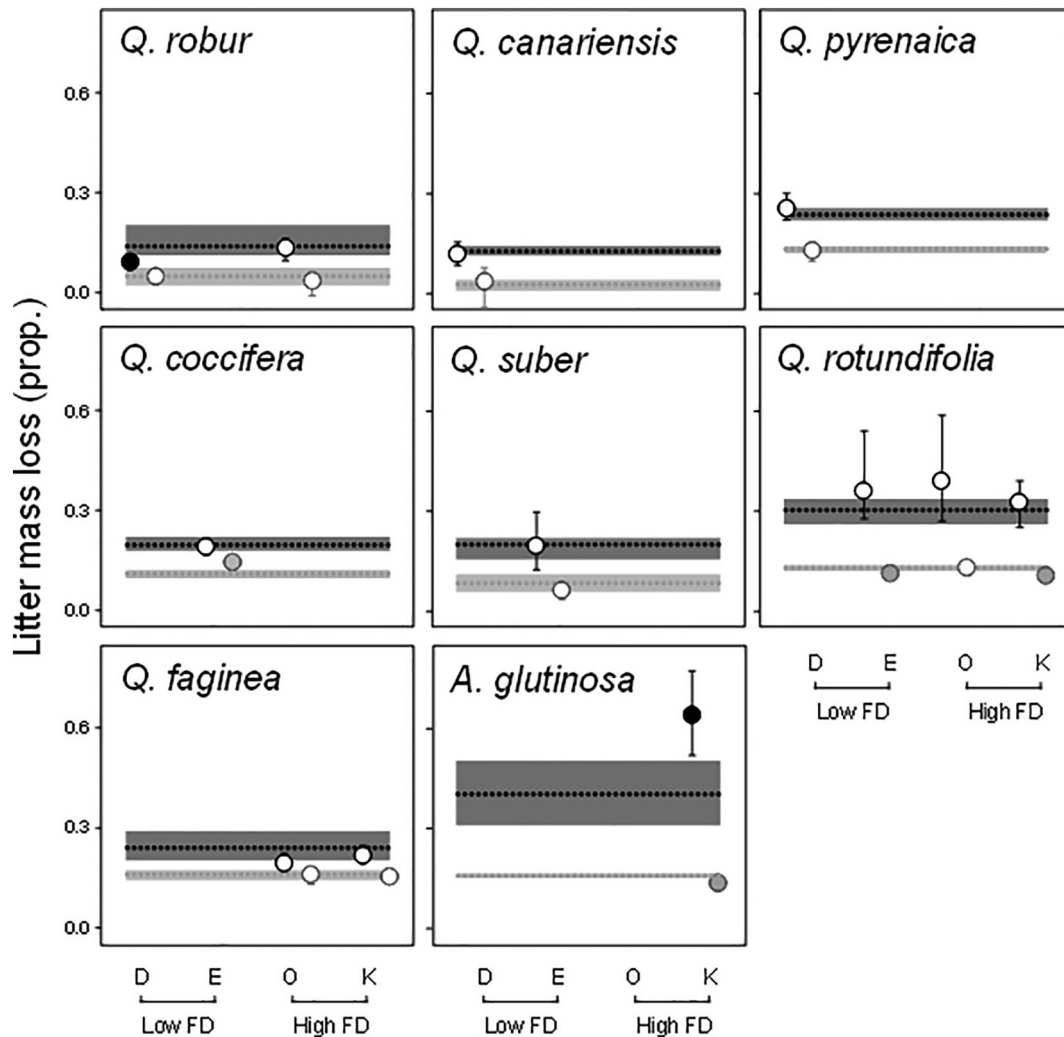
Fig. 3. Comparison of litter mass loss (LML; proportion) of each litter species among treatments where present. Circles are mean values in mixtures (black and grey represent treatments with and without detritivores, respectively). Dotted lines represent monoculture values, following the same color scale. Whiskers and shadows (dark grey for treatments with detritivores) denote upper and lower limits of 95% nonparametric bootstrapped confidence intervals for mixtures and monoculture values, respectively. Closed symbols represent intervals that are significantly different of monoculture values.

3.5. Detritivore performance

In monocultures, the highest survival of detritivores was found in *Q. suber* and *A. glutinosa* microcosms, whereas the lowest was observed in 2 deciduous oaks (*Q. robur* and *Q. pyrenaica;* Fig. S5A). Within mixtures, no statistically significant differences were found (Fig. S5B).

In monocultures, DMG was the highest when fed *Q. rotundifolia* and *A. glutinosa*, and the lowest when fed deciduous oak litter (Fig. S6A), exhibiting a linear relationship with LQI ($R = 0.45$, $p < 0.010$). DNG and DPG followed a similar pattern to that of DMG (Fig. S6B, C). DNG in monocultures was also related to LQI ($R = 0.37$, $p = 0.027$). However, *Allogamus* larvae decreased their N and P body concentration regardless of the treatment (ca. 8.6% and 14.4% of reductions of N and P, respectively). NDE on DMG was positive in the high FD-K mixture (Fig. 6A; effect size: 49%), whereas no NDE was found for DNG (Fig. 6B), and DPG exhibited a positive NDE on individuals fed Low FD-D litter (Fig. 6C).

4. Discussion

Global change forecast scenarios consider the possibility of a replacement of functional groups or the loss of key plant species in riparian areas (Kominoski et al., 2013; Salinas et al., 2018). However, the effects on stream ecosystem functioning appear to be difficult to understand due to the complex trade-offs within and among decomposers exploiting mosaics of litter species (Gessner et al., 2010; Santonja et al., 2019). This study assesses this topic from a functional approach. Our results exhibit different diversity effects on the performance of detritivores and microbial decomposers, being such effects more related with the presence of the key plant species *A. glutinosa* rather than with the FD derived from the variation in leaf habits. This highlights the crucial role of N-fixing species, and point to litter nutrient concentration as driver of B-EF relationships (Flynn et al., 2011; Santonja et al., 2019).

4.1. Functional diversity and key plant species increase trait variability of litter mixtures

The lack of consensus about which is the most suitable measure of FD in leaf litter (Lecerf et al., 2011; Petchey et al., 2009) has prevented a comprehensive assessment of its effects on stream ecosystem processes. Here, by mixing species from different functional groups (based on their leaf habits and N-fixing strategies) we confirmed the increasing trait variability along with FD, especially when *A. glutinosa* was present. That is, although FD related to leaf habits increased trait variability, it was the inclusion of the key species what made the difference. Although previous studies have attributed the observed mixing effects
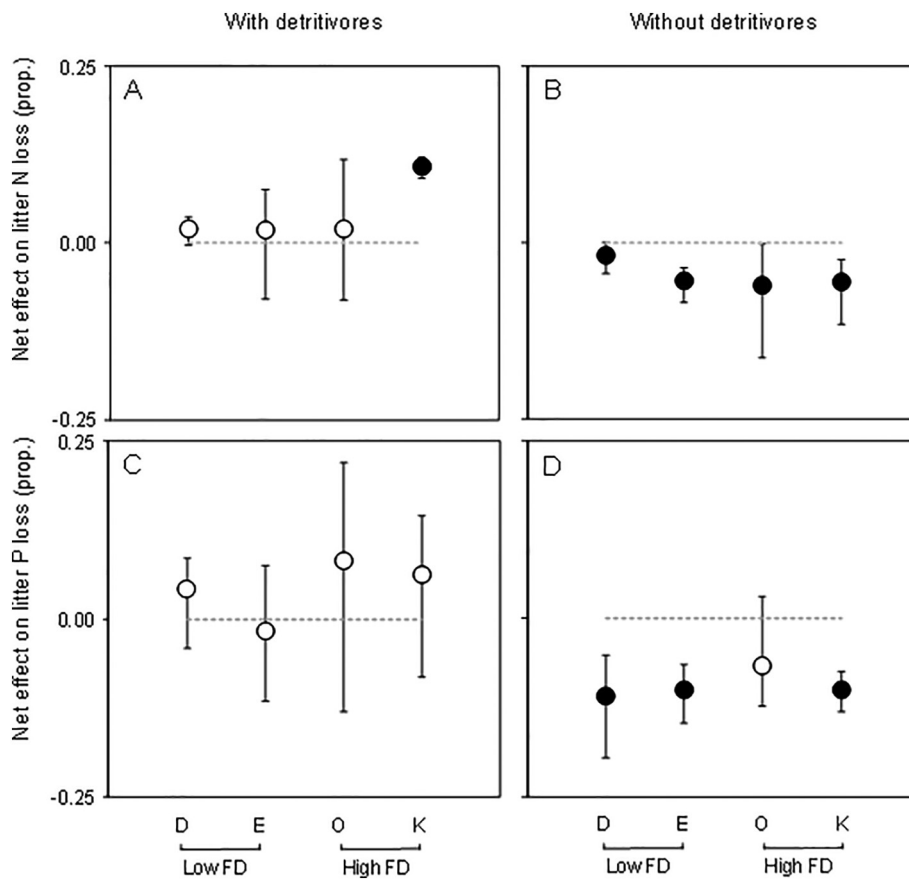
Fig. 4. Net diversity effect on litter nutrient loss (LNL and LPL; proportion). Litter N loss (A and B) and litter P loss (C and D); in microcosmos with detritivores (A and C) and without them (B and D). Mean values (circles) and upper and lower limits of 95% nonparametric bootstrapped confidence intervals (whiskers) are presented. Dashed lines denote no-effect. Closed and open circles represent intervals that reject or accept the null hypothesis, respectively.

to an increase in functional dissimilarity (e.g., Santschi et al., 2018; Vos et al., 2013), others (e.g., Santonja et al., 2019) pointed to species-specific effects through increasing mean nutrient concentrations of litter mixtures (mass ratio hypothesis; Grime, 1998). Since species phylogenetically closer are expected to share more traits, some authors (Boyero et al., 2016; López-Rojo et al., 2020) have used phylogenetic distance as a proxy of trait diversity. Yet, our results concerning oak species show that even congeneric taxa can greatly differ in relevant functional traits. Nonetheless, whatever the approach, functional diversity or phylogenetic distance, the pivotal role of both litter quality and trait variability explaining biodiversity-litter decomposition interactions, often emerges (Handa et al., 2014; Lecerf et al., 2011; López-Rojo et al., 2020).

4.2. Detritivore- and microbially-mediated decomposition respond differently to litter functional diversity

In agreement with previous studies, our results reveal a trend of increasing litter total decomposition with FD (Lecerf et al., 2011; Tonin et al., 2017). However, whereas Tonin et al. (2017) reported positive net diversity effects on both detritivore- and microbially-mediated decomposition, although of lower magnitude on the second; here we detected positive net diversity effects on detritivore- and negative on microbially-mediated decomposition, but only in the mixture with the highest diversity of traits and containing alder leaf litter (i.e. High FD-K). This, and similar findings (Larrañaga et al., 2020; López-Rojo et al., 2018), point to the existence of a threshold of litter quality dissimilarity above which the effects of diversity arise. The net diversity effect on litter decomposition was mainly explained by complementarity effects, in

agreement with previous studies (Handa et al., 2014; López-Rojo et al., 2018): positive (although not significant) in presence of detritivores, and negative for microbially-mediated decomposition. Often, positive complementarity effects lead to positive diversity effects on detritivore-mediated decomposition (e.g., Larrañaga et al., 2020). Here, positive diversity effects emerged from concomitant positive complementarity and selection effects in the mixture with *A. glutinosa*, the latter effect exhibiting its highest value when detritivore-mediated decomposition was isolated. *Allogamus* fed preferentially on the more palatable litter (selection), without altering consumption of other resources, as previously observed in field (Sanpera-Calbet et al., 2009) and microcosm experiments (Tonin et al., 2017). This contrasts with the general assumption that high-quality litter enhances decomposition of concurrent low-quality one (Gartner and Cardon, 2004), but supports the 'clutching at straws' effect (sensu Landeira-Dabarca et al., 2019), i.e. the presence of relatively recalcitrant litter, may enhance overall mixture consumption despite the reduction of average mixture quality. This emphasizes detritivore capability to feed simultaneously on resources of contrasting quality in order to balance their diet (Carvalho and Graça, 2007; Leroy and Marks, 2006).

The positive selection effect found on microbial decomposition suggests substrate preference (Gulis, 2001), however, contrary to our expectations, this selection effect was not translated into a greater decay of alder litter than that of oaks as usually reported (e.g., Cornejo et al., 2020; Monroy et al., 2016). Yet, the observed negative diversity effect on microbial decomposition was driven by negative complementarity. Three main factors could have conditioned this negative effect in our microcosms: low microbial colonization of litter as a consequence of the short incubation period, nutrient limitation (Ferreira et al., 2015)

With detritivores   Without detritivores
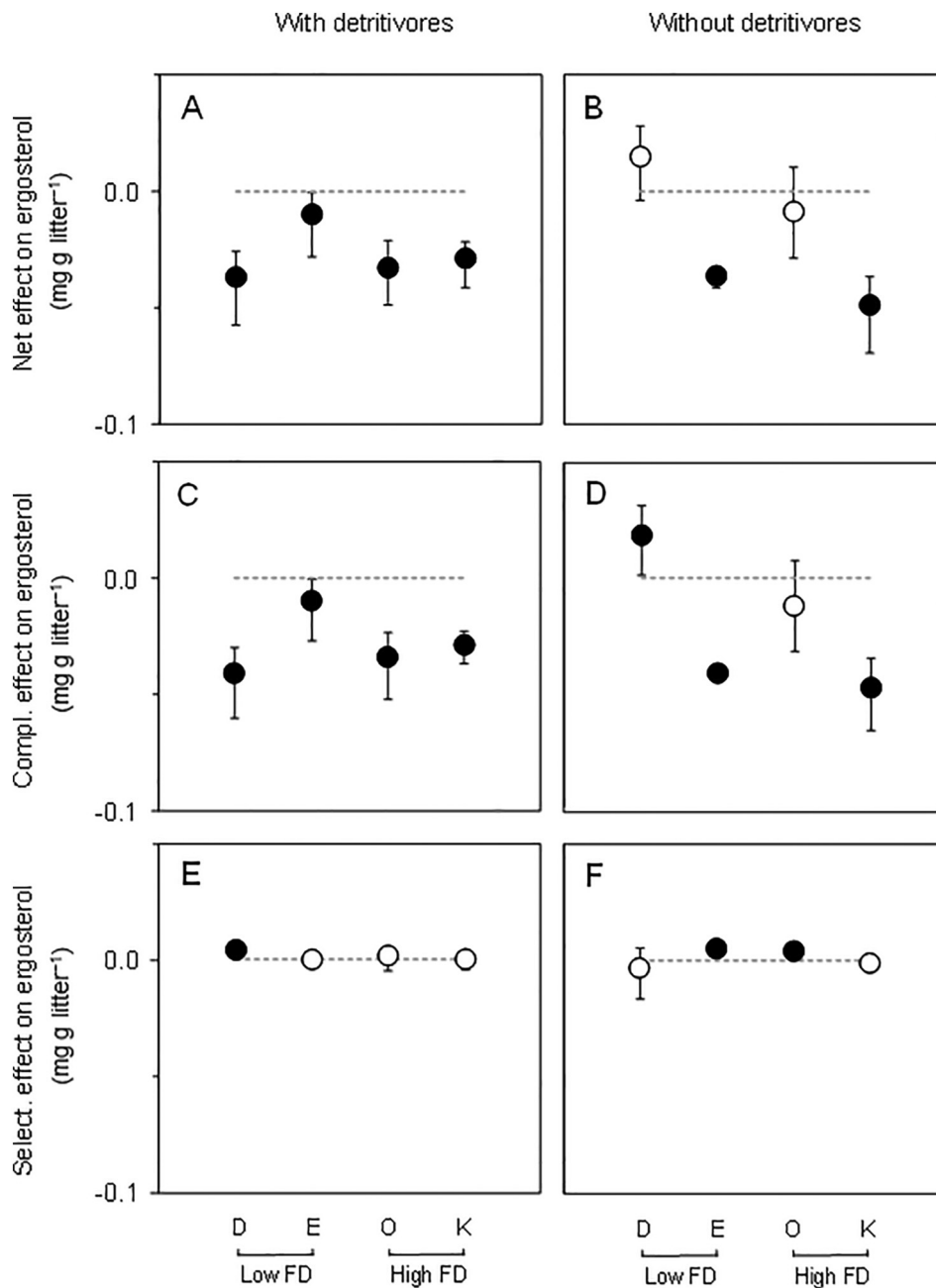
Fig. 5. Net diversity, complementarity and selection effects on fungal biomass (Ergosterol content; mg g litter^{-1}): with detritivores (A, C and E) and without detritivores (B, D and F). Mean values (circles) and upper and lower limits of 95% nonparametric bootstrapped confidence intervals (whiskers) are presented. Dashed lines denote no-effect. Closed and open circles represent intervals that reject or accept the null hypothesis, respectively.

caused by the low amount of dissolved nutrients incorporated with water renewal, and/or the accumulation of leached secondary compounds, common in oak litter (McArthur et al., 1994), with putative toxic effects on microbes (Canhoto et al., 2013). However, the latter seems more likely, since the initial colonization and the nutrient limitation appeared to be the same in all microcosms, whereas the toxic effects of accumulated soluble secondary metabolites could have differed between monoculture and mixtures treatments. These effects are more likely to be detected in microcosms with lower water renewal than in field conditions where leaf leachates are constantly washed out, and can be potentiated with increasing mixture diversity, as more different compounds coming from diverse litters may create a toxic cocktail, as defined in allelopathy studies (Reigosa et al., 1999).

4.3. Functional diversity differently affects litter nutrient recycling by detritivores and microbial decomposers

We found a general trend of accelerated nutrient dynamics with detritivores as FD raised, as reported before in large-scale field (Handa et al., 2014) and microcosm (López-Rojo et al., 2019) studies. Here, significant positive diversity effects occurred only for N cycling in the mixture with *A. glutinosa*; this exhibited, although not significantly, higher litter-N loss rates than the best performing species, supporting the premise of the limiting role of N for litter consumer detritivores in headwater streams (Balseiro and Albariño, 2006; Fenoy et al., 2020; McManamay et al., 2011). Thus, litter N variability seems to drive N loss (García-Palacios et al., 2017) and, subsequently, diversity effects (López-Rojo et al., 2020) on leaf litter decomposition.
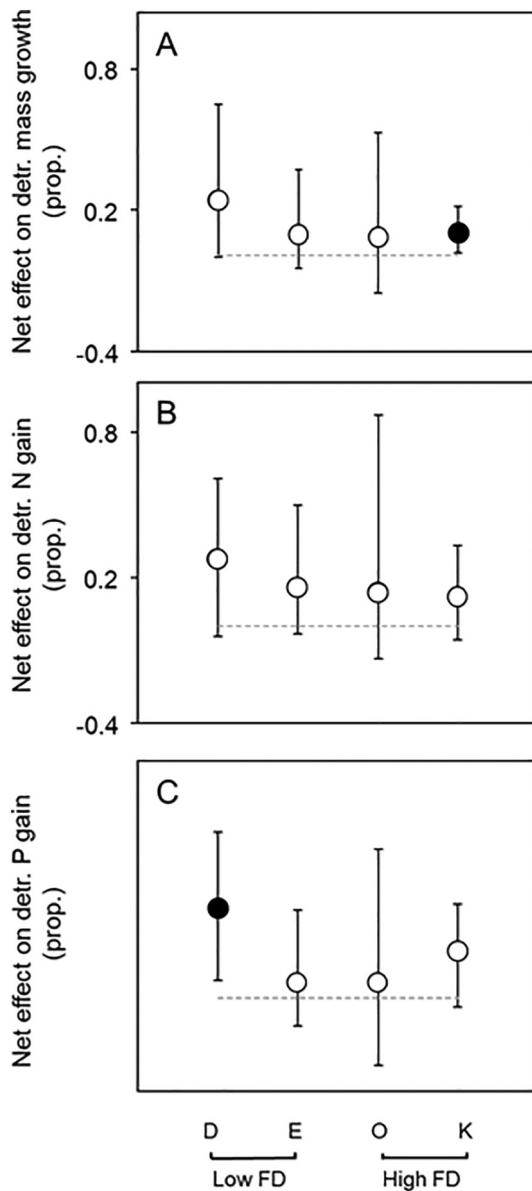
Fig. 6. Net diversity effect on detritivore growth (mass: DMG; proportion) and elemental gain (N: DNG, and P: DPG; proportion). Mean values (circles) and upper and lower limits of 95% nonparametric bootstrapped confidence intervals (whiskers) are presented. Dashed lines denote no-effect. Closed and open circles represent intervals that reject or accept the null hypothesis, respectively.

Microbially-mediated nutrient dynamics were also determined by FD and showed mostly significant negative diversity effects, i.e. mixtures lost less nutrients than expected from monocultures. However, N and P net losses (mineralization) occurred in the two mixtures with higher FD, and nutrient sequestration (immobilization) appeared in low FD mixtures, in which microbes might have used nutrients from the water (López-Rojo et al., 2019; Suberkropp and Chauvet, 1995; Tonin et al., 2017). The latter process is expected to be higher in microcosms with detritivores, due to increased nutrient release to water (López-Rojo et al., 2019; Tonin et al., 2017), but might be masked by the high detritivore-mediated decomposition. Thus, similar negative diversity effects resulted from opposed underlying mechanisms: nutrient sequestration in low FD mixtures and nutrient mobilization in high FD ones. Most likely, the negative diversity effect (negative complementarity) also observed for microbially-mediated decomposition (i.e. microbial inhibition due to the leaching of secondary compounds; see

Section 4.2) must underlie the corresponding effects on nutrients, but the specific mechanisms need further study.

4.4. Fungal biomass and detritivore performance in response to functional diversity

Contrary to our expectations (e.g., Kominoski et al., 2009), fungal biomass was, in general, negatively affected by FD. A plausible explanation for such negative diversity effect is the leaching of toxic compounds from oaks, as discussed in Section 4.2. Furthermore, high FD mixtures might promote fungal species richness (e.g., Fernandes et al., 2013) and, therefore, competitive interactions (Gessner et al., 2010; Gulis and Stephanovich, 1999), resulting in negative complementarity effects. Nevertheless, the potential limitations that our microcosms may have imposed to fungal performance (i.e., nutrient limitation and poor fungal colonization due to the short pre-incubation period of litter and the relatively lack of water renewal) could have distorted diversity effects on fungal biomass. Nonetheless, a comparison of ergosterol concentrations in alder leaf litter between our experiment (monocultures) and a field experiment carried out in streams of Sierra Nevada (Casas et al., 2011) revealed very similar values. Whatever the cause, our results contrast with other studies which found that litter mixtures may improve nutrient availability, and thus microbial performance (Handa et al., 2014; Schimel and Hättenschwiler, 2007), failing to support a positive effect of plant diversity on microbial decomposers.

Survival of detritivores was variable and appeared to depend on intraspecific interactions (Boyero and Pearson, 2006) coupled with litter quality, more than litter diversity. We observed cannibalism, likely promoted in those mixtures lacking a litter type that fulfils the nutrient requirements of detritivores (e.g., Bastian et al., 2008). Detritivore mass growth followed a similar pattern to that of detritivore-mediated decomposition, which often increased with litter quality and diversity (Fernandes et al., 2015). However, similarly to other studies (e.g., Tonin et al., 2017), detritivore growth did not increase with increasing litter diversity, although a slight trend of higher mass and nutrient gain with increasing litter FD was discerned, giving some support to the balanced diet hypothesis. Diverse mixtures provide more varied nutritional resources, which effects translate into higher consumer biomass (DeMott, 1998), what could subsequently shift from a bottom-up into a top-down control of the process (Duffy et al., 2007). This trend was, nonetheless, not entirely clear since significant positive diversity effects on growth (ca. 50%) were only detected in the highest FD treatment (High FD-K). The combination of two nutrient-rich litter species (*A. glutinosa* and *Q. rotundifolia*) in this mixture clearly favored litter consumption (Santonja et al., 2019; Vos et al., 2013) and detritivore growth (Danger et al., 2013). Cannibalism may have altered N and P gains to some extent. However, *Allogamus* larvae were only able to significantly gain N and P when fed two monocultures where no signals of cannibalism were observed and containing the most nutrient-rich litters (i.e. *A. glutinosa*-N and *Q. rotundifolia*-P), possibly because no other litter type fulfilled their, usually high, nutrients demands. N is used by caddisflies to produce somatic proteins, silk, and N-rich chitin for case building (Frainer et al., 2016), while P is required in ribosomal RNA (Sterner and Elser, 2002). Consumption of litter assemblages lacking a nutrient-rich litter may lead to a decrease in detritivore growth, which can cause a reduction of adult size (Carvalho and Graça, 2007; Flores et al., 2014), consequently hindering their reproductive success and survivorship and pointing to bottom-up effects (Martínez et al., 2013; Swan and Palmer, 2006). Despite all the aforementioned, it is important to consider that our results may not be applicable to other detritivore species, e.g., if they were more sensitive to leached secondary compounds or unable to face tough and recalcitrant leaves such as those from oaks; nor to the entire detritivore community present in the streams due to interspecific interactions.

4.5. Detritivores and A. glutinosa: key players of diversity effects on decomposition

Direct assessment of mixing effects on a particular plant species in different mixtures, allowed us to delve into mechanisms driving net diversity effects beyond complementarity and selection effects (Kominoski et al., 2009). This approach further supports the determinant role of *A. glutinosa* mediating diversity effects. In the presence of detritivores, the species accompanying *A. glutinosa* in the High FD-K treatment (*Q. rotundifolia* and *Q. faginea*) decomposed at a similar rate than in their monocultures. Therefore, suggesting that the observed significant net diversity effect found in this mixture relied mainly on an increased consumption of *A. glutinosa* within the mixture compared to its monoculture. The scarcity of the preferred litter within an assemblage may enhance the consumption of the co-existing resources (Bastian et al., 2008). Nonetheless, we provided *A. glutinosa* litter in excess (>50% of *A. glutinosa* litter remaining at the end of the experiment), what further supports the diversity effect found in our experiment. In contrast, microbially-mediated decomposition reveals the opposite pattern: nutrient-rich litters (*A. glutinosa* and *Q. rotundifolia*) exhibited lower decomposition rates in the High FD-K mixture than in monocultures, driving the observed negative diversity effect. This is possibly due to short initial litter colonization period carried out, the leaching of secondary compounds (as discussed in Section 4.2), or because positive diversity effects on microbially-mediated decomposition require longer incubation times (e.g., Fernandes et al., 2013; Santschi et al., 2018).

5. Conclusions

Our outcomes suggest combining the use of functional diversity and leaf quality when assessing B-EF relationships. This study underlines that litter nutrient content had greater relevance for diversity effects compared to diversity of other litter traits. N-fixing plant species play a prominent key role, entailing a leap of quality and trait variability of litter assemblages, which drives diversity effects on ecosystem functioning. Moreover, our results underscore the combined role of detritivores and plant key species supporting positive diversity effects on instream decomposition in headwaters. However, these outcomes must be interpreted cautiously given the difficulty to conciliate the required time to decompose of microorganisms and shredders, and the usual constraints of microcosms studies; for example, the use of only one stream detritivore species, and the fact that the microbial community was reduced to that present in the weekly water renewal, added to the possible existence of a nutrient limitation and an accumulation of litter leachates. Considering the forecasted loss of deciduous in favor of evergreen plant species (Kominoski et al., 2013; Salinas et al., 2018) and, in particular, the black alder dieback due to an emergent fungal disease (Bjelke et al., 2016; Gibbs et al., 1999), further studies should be carried out to improve our knowledge on effects of such changes and to advice managers about which species should receive special attention in conservation programs, in order to mitigate instream effects of functional changes in riparian plant communities.

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CRediT authorship contribution statement

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149266.

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