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Title: Eutrophication modulates plant-litter diversity effects on litter decomposition in streams

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Abstract

Although freshwater ecosystems are severely impacted by changes in riparian vegetation and eutrophication, their interactive effects on litter decomposition and associated biota in streams remain poorly understood. In this study, 5 leaf species were placed in coarse-mesh bags alone or in mixtures and immersed in 6 low-order streams along an eutrophication gradient. Leaf-litter decomposition and fungal biomass were higher in leaf mixtures than in single leaf species. Leaf diversity effects on decomposition were synergistic and increased with leaf species number. However, the positive diversity effects were only found in streams with lower nutrient levels, suggesting that oligotrophic streams depend more on the number of riparian plant species than eutrophic streams. On the other hand, leaf species identity affected leaf-litter decomposition, and fungal and invertebrate biomasses on leaves. A positive linear relationship between initial leaf N concentration and leaf-litter decomposition was found, and this relationship became stronger as eutrophication increased. This suggests that leaf-litter decomposition depends more on the quality than the number of riparian plant species in eutrophic streams. Overall results highlight that eutrophication modulates leaf diversity effects on leaf-litter decomposition with potential implications for stream ecosystem management.

Key words: eutrophication, leaf diversity, leaf quality, decomposition, streams, aquatic fungi, invertebrates

Introduction

Aquatic ecosystems are particularly vulnerable to global change and freshwaters are currently among the most endangered ecosystems in the world (Malmqvist and Rundle 2002, Dudgeon et al. 2006). Anthropogenic activities have conducted to a severe degradation of water resources, and water pollution was identified as a major threat to human water security and river biodiversity (Vörösmarty et al. 2010). Nutrient loading comes out as a dominating source of pollution (Vörösmarty et al. 2010), which has been increasing in rivers over the past century (Malmqvist and Rundle 2002). Besides water pollution, changes in land use, mainly due to intensification of agriculture and expansion of urban settlements, are greatly degrading forests throughout the world by altering species composition or decreasing plant diversity in riparian corridors (Graça et al. 2002, Foley et al. 2005, Haines-Young 2009).

In headwater-forested streams, plant detritus from riparian vegetation is the main source of food and energy to aquatic biota (Vannote et al. 1980). In these ecosystems, leaf-litter decomposition is a key process driven by microbes, mainly fungi, and invertebrates (Graça and Canhoto 2006, Gessner et al. 2007). A recent study across 100 European streams showed that leaf-litter decomposition was stimulated by increasing nutrient concentration until a certain level above which the process became inhibited (Woodward et al. 2012). On the other hand, changes in riparian plant diversity may result in alterations in the quantity or quality of litter inputs to streams (Webster et al. 1990). This may affect the food resources available to aquatic biota dependent on plant detritus (Webster et al. 1990, Pozo et al. 1997) leading to shifts in the structure of aquatic communities (Bärlocher and Graça 2002, Kominoski and Pringle 2009) and/or leaf-litter decomposition rates (Kominoski and Pringle 2009, Kominoski et al. 2011). A compilation of manipulative studies on the effects of plant-litter diversity on litter decomposition showed that 44% of litter mixtures decomposed faster than predicted from the sum of single litter species (synergistic effects) and 39% of litter mixtures

decomposed slower than expected from individual species decomposition (antagonistic effects, Lecerf et al. 2011). Moreover, environmental context might change the magnitude or direction of leaf-litter diversity effects. For instance, antagonistic effects of leaf-litter diversity on leaf decomposition were found in summer but not in autumn (Swan and Palmer 2004), and the antagonistic effects were also suppressed by nutrient enrichment in the stream water (Rosemond et al. 2010). Although changes in riparian diversity and nutrient concentrations in streams may occur simultaneously, their interactive effects on leaf-litter decomposition and associated aquatic biota remain poorly understood (but see, Rosemond et al. 2010).

In this study, we assessed the effects of riparian plant diversity (species quality and number) and eutrophication on leaf-litter decomposition and the associated decomposer communities in streams. For that, leaves of 5 plant species common in the riparian area of the study sites (alder, chestnut, eucalyptus, plane tree and oak) were enclosed alone or in mixtures in coarse-mesh bags and immersed in 6 streams along an eutrophication gradient. We tested: i) if leaf-litter decomposition and decomposer activity depended more on plant species number or quality; ii) if putative diversity effects could be predicted by comparing leaf-litter decomposition in plant mixtures with that expected from the weighted sum of individual plant species (additive model); and iii) how eutrophication would modulate leaf-litter diversity effects. Leaf-litter diversity effects on litter decomposition were expected to be positive because highly diverse litter mixtures would provide more diverse resources and a more stable habitat to microbial decomposers and invertebrate detritivores. Because microbes can obtain nutrients from both leaf litter and stream water (Suberkropp and Chauvet 1995), eutrophication was expected to affect litter-associated microbial activity and alter litter nutrient content, and consequently its palatability to invertebrate detritivores, potentially attenuating positive diversity effects of leaf litter.

Methods

Study sites

Field experiments were conducted at 6 stream sites of the Ave River basin (Northwest of Portugal). The Agra Stream is near the spring of the Ave River (Serra da Cabreira) in an area with low human influence. Riparian vegetation was dominated by *Castanea sativa* Mill. and *Quercus* sp. and the stream substrate was composed by boulders and pebbles. Oliveira, Andorinhas and Agrela streams are in an area with some agricultural activities. In the Oliveira Stream, the riparian vegetation was composed by *Alnus glutinosa* (L.) Gaertn., *Quercus* sp., *Platanus* sp. and *C. sativa*, and the streambed was constituted by boulders, pebbles and gravel. The Andorinhas Stream was bordered by *A. glutinosa*, *Quercus* sp. and *C. sativa* and the streambed was composed by sand and gravel. The Agrela Stream was bordered by *A. glutinosa*, *Quercus* sp. and *Eucalyptus globulus* Labill.; sand and silt dominated the substrate and boulders were also present. The Selho River is near the city of Guimarães. At the study site, the stream was bordered by *Populus* sp. and *A. glutinosa*, and the substrate was constituted by sand, gravel and boulders. The Couros Stream crosses the city of Guimarães and, at the study site, was bordered by agricultural fields and occasionally by *Populus* sp.; the streambed was dominated by sand.

Experimental setup

Leaves of *A. glutinosa* (alder, A), *C. sativa* (chestnut, C), *Platanus* sp. (plane tree, P), *Quercus robur* L. (oak, O) and *E. globulus* (eucalyptus, E) were collected just before abscission in autumn 2009, air-dried and stored until used. Leaf species were placed in plastic mesh bags (5-mm mesh; 30 x 23 cm) alone (A, C, P, O, E) or in selected combinations of 2 species (A+O, A+E, A+C), 3 species (A+O+E, A+O+C, A+E+P) and 5 species (All), in a total of 12 treatments (4 replicates per treatment). Leaves were weighed in groups of 4 ±

0.001 g. In mixtures, leaf mass was proportionally divided by the number of leaf species in each treatment (2 g, 1.33 g and 0.8 g for mixtures of 2, 3 and 5 species, respectively). Leaf bags were immersed at each stream site on 10th November 2010. After 38 days, leaf bags were retrieved, placed individually in plastic bags, and transported in a cool box (4 ºC) to the laboratory. In the laboratory, leaf litter was removed from each bag and rinsed with tap water over an 850-µm-mesh sieve to retain invertebrates. Leaf material was cut into 12-mm disks and used to estimate fungal biomass and induce fungal sporulation. The remaining leaf material was used to estimate leaf mass loss and nutrient concentration in leaves.

Physical and chemical analyses of the stream water

Physical and chemical parameters of the stream water were measured for 4 times during the study period at each sampling site. Conductivity and dissolved oxygen were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW). Stream water samples were collected in plastic bottles, transported in a cool box and used (within 24 h) for chemical analyses. Concentrations of $N\text{-}NO_3^-$ (HACH kit, cadmium reduction method, LR), $N\text{-}NO_2^-$ (HACH kit, diazotization method, LR), $N\text{-}NH_3$ (HACH kit, salicylate method) and $P\text{-}PO_4^{3-}$ (HACH kit, ascorbic acid method) in stream water were measured using a HACH DR/2000 photometer (Hach company, Loveland, CO, USA) according to the manufacturer instructions.

Identification of fungal spores and quantification of mycelial biomass

Fungal sporulation was induced by aeration of 5 leaf disks from each replicate bag in 75 mL of filtered stream water for 48 ± 4 h, at 16 ºC. Appropriate aliquots of conidial suspensions were filtered (0.45-µm-pore size, Millipore), and conidia were stained with 0.05% cotton blue in lactic acid. At least 300 spores per filter were identified and counted under a light microscope to determine the contribution of each aquatic hyphomycete species to the total

conidial production in assemblages. Fungal sporulation rates were calculated for each species as the number of spores released per gram of leaf dry mass per day.

Mycelial biomass was estimated from ergosterol concentration on leaves (Gessner 2005). Lipids were extracted from sets of 5 leaf disks by heating (80 °C, 30 min) in 8 g/L KOH in methanol, purified by solid-phase extraction and eluted in isopropanol. Ergosterol was quantified by high performance liquid chromatography (Beckmann Gold System) using a LiChrospher RP18 column (250 × 4 mm, Merck). The system was run isocratically with HPLC-grade methanol at 1.4 mL/min and 33 °C. Ergosterol was detected at 282 nm and quantified based on a standard curve of ergosterol in isopropanol (Sigma). Ergosterol concentration was converted to fungal biomass assuming 5.5 µg ergosterol/mg mycelial dry mass (Gessner and Chauvet 1993).

Invertebrate identification and biomass

Leaf-associated invertebrates were preserved in 96% ethanol and identified to the lowest possible taxonomic level (Tachet et al. 2010). After identification and counting, invertebrates were dried at 80 °C to constant mass (72 ± 24 h) and weighed to the nearest ± 0.0001 g.

Leaf mass loss

The remaining leaf litter was freeze-dried to constant mass (72 ± 24 h) and weighed (± 0.0001 g). Leaf mass loss was estimated as the difference between leaf mass at the beginning and at the end of experiment. Additional leaf bags from the 12 treatments were freeze-dried to constant mass and weighed to determine the conversion factor between air-dried mass and freeze-dried mass of leaves.

Nitrogen concentration in leaves

Portions of leaf material (ca. 120 mg) were ground and used to estimate initial and final concentration of nitrogen (N) in each leaf litter treatment. Nitrogen concentration in leaf litter was determined in a LECO-CNS 2000 Elemental Analyzer (Leco Corp., St. Joseph, MI, USA) at the Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI, University of Vigo, Spain).

Statistical analyses

A principal component analysis (PCA) was used to ordinate sites according to nutrient concentrations, oxygen concentration and conductivity in the stream water (CANOCO version 4.5, Microcomputer Power, Ithaca, NY).

Nested ANOVAs were used to test the effects of leaf species number, identity (nested within species number) and stream eutrophication on leaf mass loss, and fungal and invertebrate biomasses (Zar 2009). Because the experimental design was unbalanced, we applied Type III analyses of variance with the Variance Estimation and Precision (VEPAC) module in STATISTICA 8.0 (Statsoft, Tulsa, OK, USA). Differences between treatments were analyzed with Tukey-Krammer´s post-tests (Zar 2009).

Leaf diversity effects on decomposition were further assessed as deviation from additivity, i.e. as the difference between observed leaf mass loss in mixtures and expected values based on the sum of individual leaf species mass loss weighed by their proportion in the mixture (Duarte et al. 2006). Differences between observed and expected mass loss were tested against the null hypothesis that the average difference equaled 0 (t-test) (Duarte et al. 2006). Linear regressions were used to establish the relationship between initial % N of leaves and leaf mass loss in each stream. Differences among streams were compared by ANCOVA, followed by Tukey's tests (Zar 2009).

Nitrogen immobilization (N_m) was calculated as the difference between final (N_f) and initial N concentration (N_i) of leaves and expressed as: $N_m=((N_f-N_i)/N_i)100$. Values of nitrogen immobilization on leaves were tested against the null hypothesis that the average equaled 0 (t-test). A 1-way ANOVA was used to test the effect of leaf identity or stream eutrophication on N immobilization followed by a Tukey's test (Zar 2009).

To assess how leaf species identity influenced the assemblages of aquatic hyphomycetes (no. of spores produced by each fungal species per gram of leaf dry mass per day) and invertebrates (no. of individuals of each species per leaf bag), data were subjected to multidimensional scaling ordination (MDS) (Clarke and Warwick 2001). Data were then subjected to Unweighted Pair-Group Method Average (UPGMA) cluster analysis, and the result was superimposed in the MDS plot (Clarke and Warwick 2001). Prior to ordination and cluster analyses, data were log (x+1) transformed and converted into a Bray Curtis similarity matrix.

MDS and cluster analyses were done in PRIMER v6 (Software package; Plymouth Marine Laboratory, Plymouth, UK) and the other statistical analyses were done in STATISTICA 8.0 for Windows (Statsoft, Tulsa, OK, USA).

Results

Physical and chemical characteristics of the stream water

Nutrient concentrations in the stream water at the 6 sampling sites varied as follows: 0.16--3.36 mg/L N-NO_3^-, 0.005--0.18 mg/L N-NO_2^-, 0.005--3.65 mg/L N-NH_3 and 0.002--0.27 mg/L P-PO_4^{3-}. Conductivity ranged from 16 to 324 µS/cm, while dissolved oxygen ranged from 5.9 to 11.2 mg/L. The PCA ordination of sampling sites according to the stream water variables showed that PC1 and PC2 explained 99.4% of variance (Fig.1), and distributed the

streams along a gradient of increasing eutrophication as follows: Agra Stream < Oliveira Stream < Andorinhas Stream < Agrela Stream < Selho River < Couros Stream.

N immobilization in leaves

Initial N concentration in leaf litter treatments with alder (A), chestnut (C), oak (O), plane tree (P) and eucalyptus (E), alone or in mixtures, varied as follows: A (3.83%) > A+C (3.69%) > A+O (3.38%) > A+O+C (3.25%) > C (3.17%) > O (2.74%) > A+E (2.51%) > A+O+E (2.47%) > A+E+P (2.42%) = All (2.42%) > E (1.88%) > P (1.82%). During leaf immersion in streams, N immobilization occurred in all leaf treatments (t-test, $p<0.05$), except for oak leaves (t-test, $p=0.063$; Fig.2a). N immobilization in plane tree (N_m=53%) was higher than in other leaf treatments (1-way ANOVA, Tukey's test, $p<0.05$), except in eucalyptus and mixtures with all leaf species. N immobilization in leaves increased with the increase in eutrophication and was highest in the Oliveira Stream, Andorinhas Stream and Selho River (1-way ANOVA, Tukey's test, $p<0.05$; Fig.2b).

Biomass of fungi and invertebrates

Fungal biomass on decomposing leaves varied from 2.2 to 145 mg/g leaf dry mass. Stream, leaf species number and identity affected fungal biomass (nested ANOVA, $p<0.001$ for all factors; Table 1; Fig.3a--c). Fungal biomass was highest in chestnut, mixtures of alder, oak and eucalyptus, and mixtures of all leaf species (Tukey-Krammer´s test, $p<0.05$; Fig.3a). Fungal biomass was higher in mixtures of 5 leaf species than in treatments with lower species number (Tukey-Krammer´s test, $p<0.05$; Fig.3b). Fungal biomass was higher in the Oliveira Stream than in streams with lower or higher levels of eutrophication (Tukey-Krammer´s test, $p<0.05$; Fig.3c). In the most eutrophic stream (Couros Stream), fungal biomass was almost 7-times lower than in the most oligotrophic stream (Agra Stream). Significant interactions were

found between stream and leaf species identity, and stream and leaf species number (nested ANOVA, $p<0.001$ and $p=0.017$, respectively). Effects of leaf species identity on fungal biomass were stronger at moderate eutrophication levels (Oliveira, Andorinhas and Agrela streams, Tukey-Krammer´s test, $p<0.05$; data not shown).

Invertebrate biomass ranged from 1.6 to 308 mg/g leaf dry mass. Invertebrate biomass varied with the stream and leaf species identity (nested ANOVA, $p<0.001$ and $p=0.016$, respectively), but not with leaf species number (nested ANOVA, $p>0.05$; Table 1; Fig.3a--c). The highest invertebrate biomass was associated with alder and chestnut leaves (Fig.3a). Invertebrate biomass was lower in the Agra Stream (Tukey-Krammer´s test, $p<0.05$; Fig.3c), tended to increase with the level of eutrophication and attained the highest value in the Selho River (Tukey-Krammer´s test, $p<0.05$). Invertebrate biomass in the Couros Stream was less than half the value observed in the Selho River (Tukey-Krammer´s test, $p<0.05$).

Leaf decomposition

Stream, leaf species number and identity affected leaf mass loss (nested ANOVA, $p<0.001$ for all factors; Table 1, Fig.3d--f). Leaf mass loss was higher for alder, mixtures of alder and chestnut, and mixtures of alder, chestnut and oak (Tukey-Krammer´s test, $p<0.05$; Fig.3d). Leaf mass loss was higher in mixtures of 2 and 3 species compared to single species treatments (Tukey-Krammer´s test, $p<0.05$), but no differences between leaf mixtures were found (Tukey-Krammer´s test, $p>0.05$; Fig.3e). Leaf mass loss was low at the most oligotrophic site (Agra Stream), increased with eutrophication until a certain level (Selho River) and then decreased at the most eutrophic site (Couros Stream) (Tukey-Krammer´s test, $p<0.05$; Fig.3f). A significant interaction was found between effects of leaf identity and stream on leaf mass loss (nested ANOVA, $p=0.023$; Table 1). Apart from the Couros Stream, leaf mass loss was positively related to initial % of N in leaves (linear regression, $p<0.05$, Fig.

4; Table 2). Moreover, the dependence of leaf mass loss on % of N in leaves increased with eutrophication as shown by the differences in the slopes of these relationships (ANCOVA, $p<0.05$).

Leaf-litter diversity effects on leaf mass loss, assessed as deviation from additivity, increased with increasing number of leaf species in mixtures (Fig.5a). Significant positive diversity effects were found in mixtures of 3 and 5 species (t-tests, $p<0.05$). Effects of leaf diversity were positive in the less eutrophic streams, namely Agra and Oliveira streams (t-tests, $p<0.05$; Fig.5b), but not in streams with higher levels of eutrophication (t-tests, $p>0.05$).

Fungal and invertebrate assemblages

The MDS ordination of aquatic hyphomycete species sporulating on leaves separated assemblages in leaf mixtures from those in single leaf species, especially those in eucalyptus leaves (Fig.6). Within leaf mixtures, fungal assemblages in mixtures containing eucalyptus leaves shared 85% of similarity and were separated from fungal assemblages in other leaf mixtures.

The MDS ordination of leaf-associated invertebrate assemblages separated assemblages in single leaf species from those in leaf mixtures, and assemblages within each of these groups shared 80% similarity (Fig.6). Within single leaf species, invertebrate assemblages associated with alder and chestnut leaves shared 85% of similarity.

Discussion

Our results suggest that riparian plant diversity have positive effects on leaf-litter decomposition mediated by fungi and invertebrates in streams. This agrees with other studies indicating that effects of leaf-litter diversity on litter decomposition are mostly positive (Gartner and Cardon 2004, Lecerf et al. 2011). In addition, we found that plant diversity

effects increased with the increase in leaf species number (Fig.5a), suggesting that the loss of riparian tree species might affect stream ecosystem functioning by weakening interactions between leaves and decomposers in leaf species mixtures. Moreover, our results showed that eutrophication could modulate diversity effects: synergistic diversity effects on leaf decomposition were observed in streams with lower nutrient levels (Agra and Oliveira Streams) but not in eutrophic streams (Fig.5b). To our knowledge, the only study carried out to assess the combined effects of nutrient enrichment and litter diversity on litter decomposition in streams found antagonistic effects of litter species mixtures on decomposition, but these effects were lost when nutrient concentrations in the stream water increased (Rosemond et al. 2010). Nevertheless, the level of litter diversity used in that study (up to 3 species) was lower than that of our study (up to 5 species) and the addition of nutrients to the stream water resulted in much lower nutrient concentrations (up to 0.39 mg/L DIN and 0.06 mg/L SRP) than those found in our eutrophic streams (up to 3.36 mg/L $N\text{-}NO_3^-$, 3.65 mg/L $N\text{-}NH_3$, 0.18 mg/L $N\text{-}NO_2^-$ and 0.27 mg/L $P\text{-}PO_4^{3-}$).

Plant-litter decomposition is expected to be faster at higher levels of litter diversity because a wider range of resources is available to litter decomposers. However, leaf litter is not the sole source of nutrients for organisms involved in leaf decomposition. Aquatic fungi obtain nutrients mainly from leaf litter, but they also have the ability of using nitrogen and phosphorus directly from the stream water (Suberkropp and Chauvet 1995). So, in streams with higher nutrient concentrations, microbes may take benefit of the available nutrients in the stream water, contributing to explain the loss of positive effects of leaf mixtures found in our eutrophic streams. Also in these streams, N immobilization, especially in lower quality leaf types such as the plane tree, may have led to a homogenization of resource quality and to a decrease of available niches, increasing competition between invertebrates and weakening the effects of leaf-litter diversity (Bastian et al. 2008).

The effects of leaf-litter diversity are frequently explained by the identity of species that constitute the mixture rather than the number of litter species (Swan and Palmer 2004, 2006, Lecerf et al. 2007). The effects of leaf-litter identity on leaf decomposition may result from differences in litter quality, such as concentration of lignin, cellulose, nitrogen or phosphorus (Lecerf and Chauvet 2008, Schindler and Gessner 2009, Fernandes et al. 2012). In our study, treatments containing leaves with higher N content, namely alder alone or in mixtures with chestnut, showed the highest leaf decomposition. Indeed, apart from the Couros Stream, a positive linear relationship between leaf mass loss and initial N concentration in leaves was observed, and this relationship became stronger as eutrophication increased (Fig.4). The strengthening of relationships between leaf-litter quality and decomposition in eutrophic streams might have occurred because fungi benefit from dissolved nutrients leading to an enhanced use of leaf carbon. Moreover, the enhanced microbial nutrient uptake from the water column (Cross et al. 2003, Gulis and Suberkropp 2003, Ferreiro et al. 2011) and the subsequent increase in fungal activity (Sridhar and Bärlocher 2000, Gulis and Suberkropp 2003, Ferreira and Chauvet 2011) in nutrient enriched streams may also contribute to improve leaf-litter quality to invertebrate shredders leading to an overall increase in leaf-litter decomposition. The lack of leaf-litter diversity effects in the most eutrophic stream (Couros Stream) might be due to the inhibition of fungal and invertebrate activity on leaves, as supported by the very low fungal biomass and the absence of shredders at this site. Indeed, in the Couros Stream, invertebrates were mainly Chironomidae (not shown), which are considerably generalists covering a wide trophic spectrum, from filtration to predation (Oscoz et al. 2011); hence, their contribution to leaf decomposition could be less dependent on leaf-litter quality.

In our study, effects of leaf-litter diversity and/or identity on leaf decomposition were accompanied by shifts in fungal and invertebrate assemblages. Fungal assemblages on single

species separated from those on leaf mixtures, and assemblages in mixtures with eucalyptus leaves further separated from the others. Eucalyptus leaves contain oils and tannic acids that inhibit the growth of aquatic fungi (Canhoto and Graça 1999) and, thus, we might expect that some fungi would avoid eucalyptus leaves or mixtures containing it. Decreased diversity of aquatic fungi and shifts in community composition were previously found in streams crossing monocultures of eucalyptus compared with streams bordered by native mixed forests (Bärlocher and Graça 2002). In our study, invertebrate assemblages in leaf mixtures also differed from those in single leaf species. Within single species, invertebrate assemblages associated with leaves with higher initial N concentration (alder and chestnut) separated from the others. These findings suggest that alterations in riparian plant diversity may change the structure of aquatic communities in detritus food webs in streams.

A closer look on the selected streams showed that they were assigned to a gradient of eutrophication defined by an increase in inorganic nutrients and a decrease in dissolved O2. Streams with moderate and moderately-high levels of eutrophication had higher leaf-litter decomposition than oligotrophic (Agra Stream) or hypertrophic streams (Couros Stream). These results agree with those describing a hump-shaped relationship between leaf decomposition and eutrophication (Woodward et al. 2012). Consistently, the biomass of fungi and invertebrates associated with decomposing leaves followed the same pattern: overall biomass increased with the eutrophication level but decreased at the most eutrophic stream (Couros Stream). At this stream, oxygen concentration in the stream water was the lowest (5.9 mg/L). Hypoxic and anoxic conditions that are usually associated with eutrophic and hypertrophic environments besides excluding invertebrate shredders (Pascoal et al. 2003, 2005) are known to inhibit microbial activity (Pascoal and Cássio 2004), further compromising plant-litter decomposition.

Overall results suggest that effects of riparian plant diversity on stream ecosystem functioning are modulated by eutrophication. Both the quality and number of plant species affected leaf-litter decomposition by microbes and invertebrates in streams. Specifically, we found synergistic effects of leaf-litter diversity on leaf decomposition. However, positive effects of leaf species number on leaf decomposition were only observed in the less eutrophic streams. This suggests that oligotrophic streams are more dependent on the number of riparian plant species than eutrophic streams. If so, riparian plant diversity should be preserved in oligotrophic systems to maintain leaf-litter decomposition. On the other hand, the positive effects of leaf-litter quality (leaf N) on leaf-litter decomposition were strengthened by increased nutrient concentrations in the stream water, suggesting that leaf-litter decomposition depends more on the quality than the number of riparian plant species in eutrophic streams. These findings lend support to our hypothesis that eutrophication modulates leaf diversity effects on leaf decomposition with potential implications to ecosystem management. Given the worldwide commitment of controlling nutrient pollution and reducing eutrophication in streams (e.g., Water Framework Directive 2000/60/EC), action plans should pay particular attention to riparian plant diversity not only to mitigate nutrient losses to aquatic ecosystems but also to avoid an intensification of effects of plant species loss on stream ecosystem functioning.

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Figure captions

Fig.1 Principal Component Analysis (PCA) of the stream water variables in 6 streams of the Ave River basin. The vector length reflects the contribution of each variable to the 2 PC axes in relation to all possible PC axes.

Fig.2 N immobilization (% of initial N) in leaves according to leaf species identity treatment (a) and stream eutrophication (b). Leaves were immersed for 38 days in 6 streams of the Ave River basin. Positive values indicate N immobilization. Mean ± 95% CI. Significant immobilization exists when the confidence intervals do not cross 0. Leaf types: A, alder; C, chestnut; P, plane tree; O, oak; E, eucalyptus.

Fig.3 Leaf-associated fungal and invertebrate biomasses (a--c) and leaf mass loss (d--f) as function of leaf species identity (a, d), leaf species number (b, e), and stream eutrophication (c, f). Leaves were immersed for 38 days in 6 streams of the Ave River basin with increasing levels of eutrophication. Black bars, invertebrate biomass; white bars, fungal biomass. Leaf types: A, alder; C, chestnut; P, plane tree; O, oak; E, eucalyptus. Mean + 1 SEM.

Fig.4 Relationship between initial N concentration in leaves and leaf mass loss after 38 days of leaf immersion in 6 streams of the Ave River basin. Data was fitted to linear regression.

Fig.5 Leaf diversity effects on leaf mass loss assessed as deviation from additivity (observed minus expected leaf mass loss) at each level of leaf species number using average data of all streams (a) and at each level of stream eutrophication using average data of all diversity levels (b). Negative deviation from additivity indicates antagonistic response (lower leaf-litter

decomposition), and positive deviation indicates synergistic response (higher leaf-litter decomposition) of litter mixtures. Mean ± 95% CI. Significant litter mixtures effects exist when the confidence intervals do not cross 0.

Fig.6 Multidimensional scaling ordination (MDS, Bray-Curtis similarity) of taxa of fungi (sporulation rate) and invertebrates (abundance) on leaves according to leaf species identity. Leaf types: A, alder; C, chestnut; P, plane tree; O, oak; E, eucalyptus. Stress values indicate that values are not randomly distributed. Circles indicate similarities within assemblages superimposed in the MDS from cluster analysis.

Table 1. ANOVAs of the effects of stream, species number and species identity (nested within species number) on fungal biomass, invertebrate biomass, and leaf mass loss.

	Effect	SS	df	*F*	*p*
Fungal biomass	Stream	6,090,445.0	5	111.63	<0.001
	Species number	375,485.0	3	11.47	<0.001
	Identity (Species number)	603,491.0	8	6.91	<0.001
	Stream*Identity (Species number)	1,066,689.0	40	2.44	<0.001
	Stream*Species number	333,221.0	15	2.04	0.017
	Error	1,571,380.0	144		
Invertebrate biomass	Stream	254.1	5	31.70	<0.001
	Species number	0.4	3	0.09	0.967
	Identity (Species number)	31.2	8	2.43	0.016
	Stream*Identity (Species number)	50.7	40	0.79	0.811
	Stream*Species number	7.8	15	0.32	0.993
	Error	339.8	212		
Leaf mass loss	Stream	34,948.1	5	53.84	<0.001
	Species number	5505.2	3	14.14	<0.001
	Identity (Species number)	17,031.1	8	16.40	<0.001
	Stream*Identity (Species number)	8134.8	40	1.57	0.023
	Stream*Species number	1805.6	15	0.93	0.535
	Error	27,912.1	215		

1 Table 2. Linear regressions of the relationship between initial N concentration in leaves and
2 leaf mass loss after 38 days of leaf immersion in 6 streams of the Ave River basin, as shown
3 in Fig.4. Slopes were compared by ANCOVA followed by Tukey's tests.

Stream	Equation	*p*	r^2	Tukey's test
Agra	Y=8.07X+5.74	< 0.0001	0.51	a
Oliveira	Y=8.92X+20.65	0.0006	0.23	b
Andorinhas	Y=13.84X+10.42	< 0.0001	0.42	b, c
Agrela	Y=13.91X+15.90	0.0004	0.24	c, d
Selho	Y=19.59X+3.13	< 0.0001	0.36	d
Couros	Y=0.20X+30.11	0.9226	<0.01	a

4 Similar letters indicate no significant differences between slopes
5

Figure 1

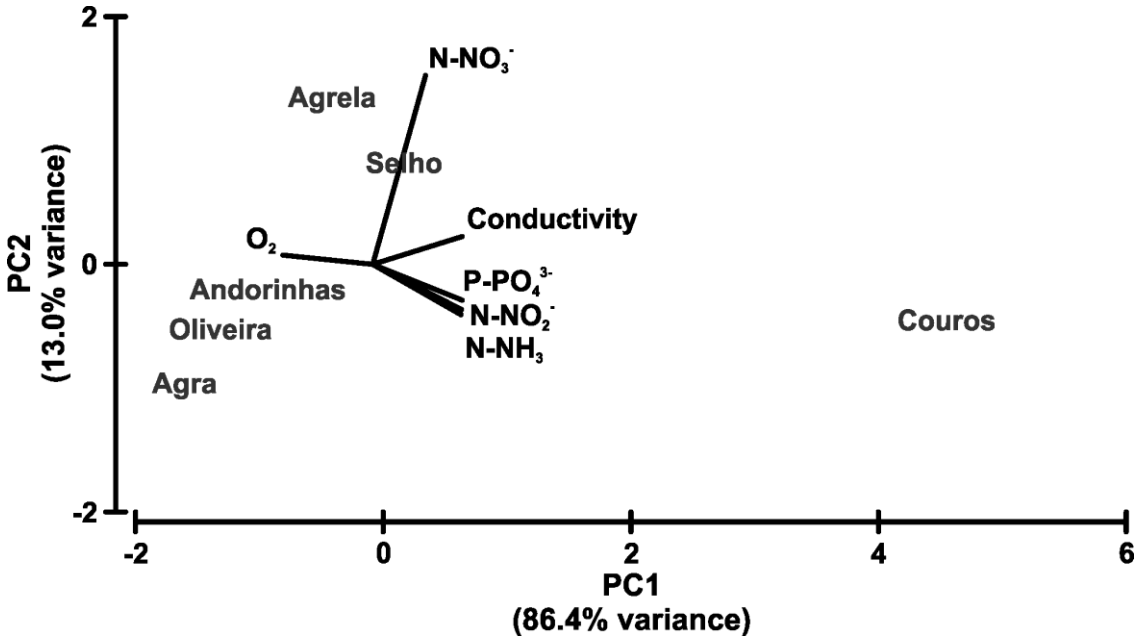

2
0
-2
PC2
(13.0% variance)
N-NO3-
Agrela
Selho
Conductivity
O2
P-PO43-
N-NO2-
N-NH3
Andorinhas
Oliveira
Agra
Couros
-2
0
2
4
6
PC1
(86.4% variance)

Figure 2

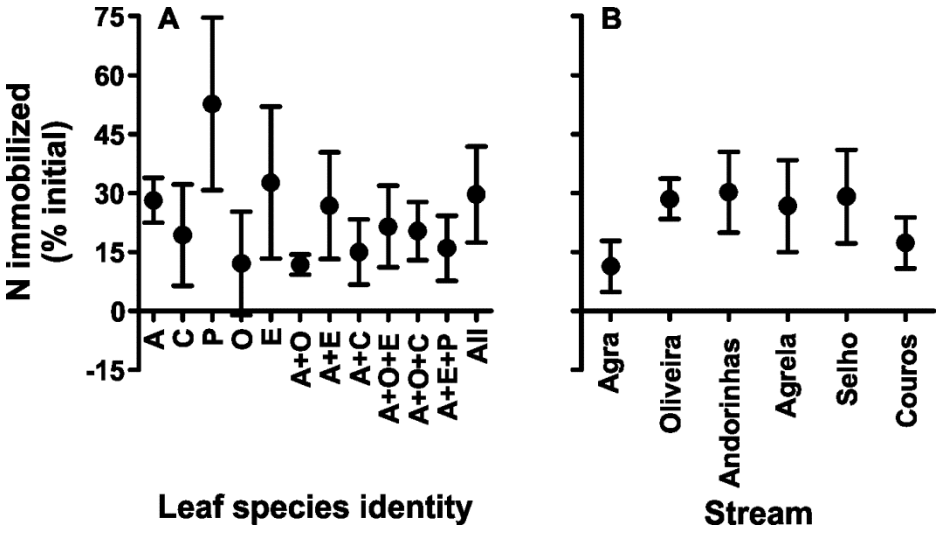

A
B
N immobilized (% initial)
75
60
45
30
15
0
-15
A
C
P
O
E
A+O
A+E
A+C
A+O+E
A+O+C
A+E+P
All
Agra
Oliveira
Andorinhas
Agrela
Selho
Couros
Leaf species identity
Stream

Figure 3

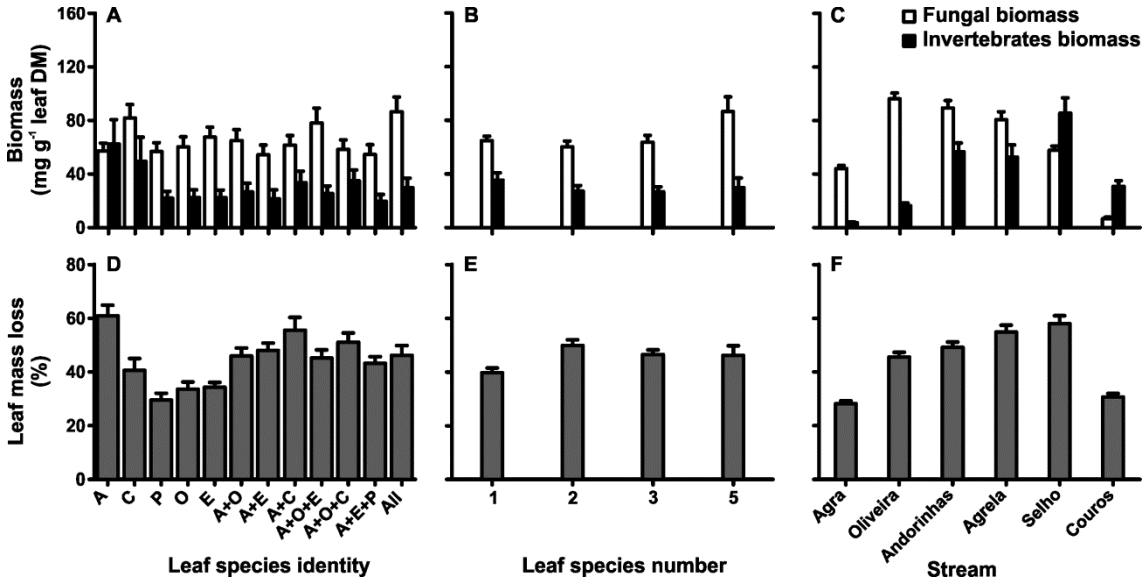

A
B
C
Fungal biomass
Invertebrates biomass
Biomass (mg g-1 leaf DM)
160
120
80
40
0
D
E
F
Leaf mass loss (%)
80
60
40
20
0
A
C
P
O
E
A+O
A+E
A+C
A+O+E
A+O+C
A+E+P
All
Leaf species identity
1
2
3
5
Leaf species number
Agra
Oliveira
Andorinhas
Agrela
Selho
Couros
Stream

Figure 4

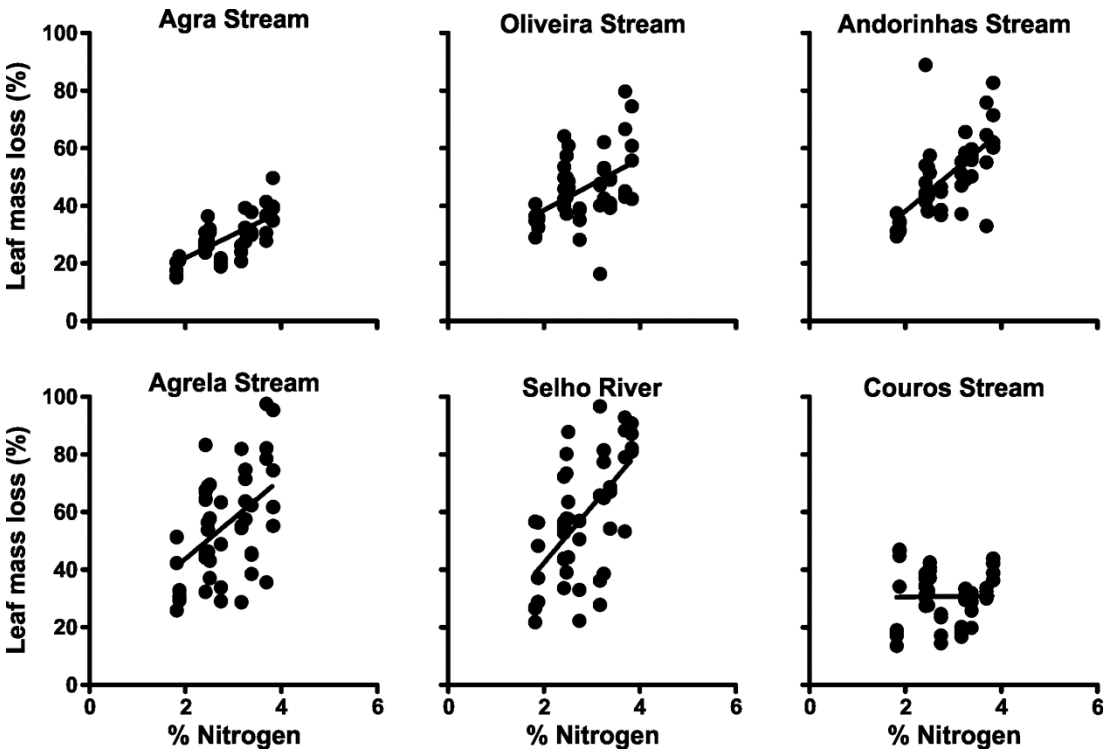

Agra Stream
Oliveira Stream
Andorinhas Stream
Agrela Stream
Selho River
Couros Stream
Leaf mass loss (%)
% Nitrogen
0
20
40
60
80
100
2
4
6

Figure 5

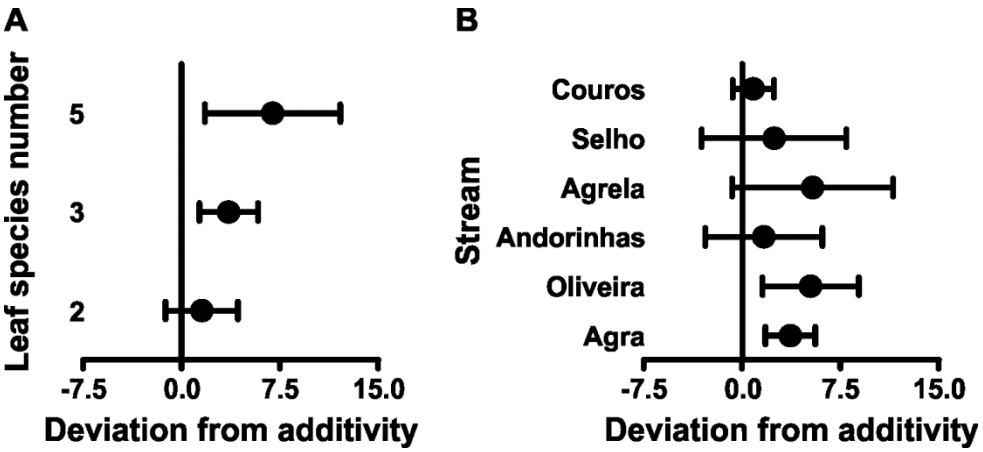
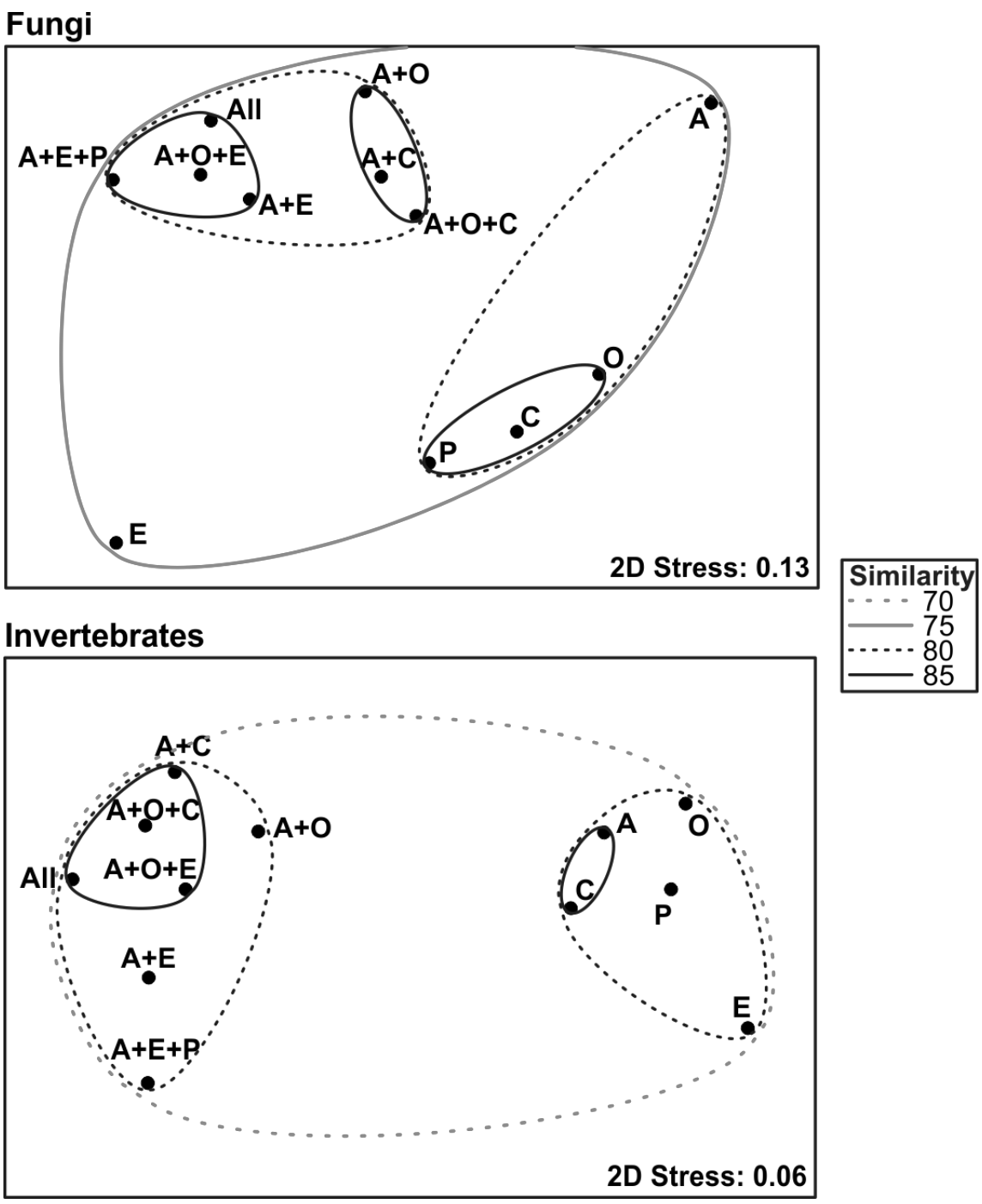

A
Leaf species number
5
3
2
-7.5
0.0
7.5
15.0
Deviation from additivity
B
Stream
Couros
Selho
Agrela
Andorinhas
Oliveira
Agra
-7.5
0.0
7.5
15.0
Deviation from additivity

Figure 6


Fungi
A+O
All
A
A+E+P
A+O+E
A+C
A+E
A+O+C
O
C
P
E
2D Stress: 0.13
Similarity
70
75
80
85
Invertebrates
A+C
A+O+C
A+O
All
A+O+E
A
O
C
P
A+E
E
A+E+P
2D Stress: 0.06