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**Eutrophication alters the effects of  
riparian plant diversity on litter  
decomposition by macroinvertebrates**

Master Thesis

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## Abstract

In low-order forested streams, plant-litter decomposition is a key ecosystem process. Invertebrate shredders are responsible for the breakdown of plant litter and are very sensitive to stream water quality degradation. Increased eutrophication and loss or alteration of riparian vegetation can have negative effects on stream organisms and alter ecosystem processes. However, the interactive effects of riparian vegetation loss and increased nutrient concentrations in the stream water are poorly understood.

In this study, we investigated if plant-litter decomposition and invertebrate assemblages were affected by leaf litter diversity and if eutrophication modulated the observed effects. Leaves from five riparian tree species (*Alnus glutinosa*, *Castanea sativa*, *Eucalyptus globulus*, *Platanus* sp. and *Quercus robur*) were used either alone or in mixtures of equally partitioned mass of 2, 3 and 5 species, in a total of 12 combinations. Leaves were placed in coarse-mesh bags and immersed in seven streams of Northwest Portugal along a gradient of eutrophication, for 38 days.

Leaf litter diversity had positive effects on litter decomposition but negatively affected invertebrate assemblages. Eutrophication modulated leaf litter diversity effects by suppressing positive diversity effects on litter decomposition and by negatively affecting invertebrate assemblages. A possible homogenization of litter nutrient content in leaf mixtures might explain the negative litter diversity effects on invertebrate communities, by promoting invertebrate unselective feeding or increasing species competition. Moreover, harmful effects of toxicant compounds, such as ammonia, might have contributed to the negative diversity effects on invertebrate assemblages in eutrophic streams. These findings illustrate that human activities alter litter decomposition dynamics in streams and the associated biotic communities.



## Resumo

Em rios de floresta, a decomposição da folhada é um processo chave para o funcionamento do ecossistema. Os invertebrados detritívoros têm um papel relevante neste processo do ecossistema e são muito sensíveis à poluição. O aumento da eutrofização e perdas ou alterações da vegetação ribeirinha podem ter consequências negativas para os organismos aquáticos e para o funcionamento dos ecossistemas de rio. No entanto, os efeitos da interacção entre a perda de vegetação ribeirinha e do aumento da concentração de nutrientes na água são pouco conhecidos.

Neste estudo investigámos se a diversidade da folhada afectaria a sua decomposição e os invertebrados associados e se a eutrofização modificaria os efeitos observados. Utilizámos folhas de cinco espécies de árvores ripícolas (*Alnus glutinosa*, *Castanea sativa*, *Eucalyptus globulus*, *Platanus* sp. e *Quercus robur*) sozinhas ou em misturas de igual proporção de 2, 3 e 5 espécies, num total de 12 combinações. As folhas foram colocadas em sacos de malha grossa e imersos em sete rios do norte de Portugal ao longo de um gradiente de eutrofização, durante 38 dias.

A diversidade da folhada afectou positivamente a sua decomposição e negativamente os invertebrados associados. O aumento da eutrofização suprimiu os efeitos positivos da diversidade na decomposição da folhada e níveis moderados a elevados de eutrofização promoveram efeitos negativos nas comunidades de invertebrados. Uma possível homogeneização do conteúdo em nutrientes nas misturas de folhas poderá ter levado a uma alimentação não selectiva dos invertebrados ou a um aumento da competição entre as espécies, o que poderá justificar os efeitos negativos da diversidade da folhada na comunidade de invertebrados. Além disso, a presença de compostos tóxicos, como a amónia, nos rios mais eutrofizados poderá ter afectado negativamente as comunidades de invertebrados. Estes resultados mostram que as actividades antropogénicas alteram a dinâmica de decomposição da matéria orgânica nos rios e as comunidades bióticas associadas a este processo.





# Table of contents

<b>1. Introduction</b>	
1.1. Biodiversity and ecosystem functioning	1
1.1.1. Mechanisms behind BEF relationships	2
1.1.2. Contribution of stream ecology to BEF research	3
1.2. Leaf litter decomposition in streams	4
1.2.1. Contribution of microorganisms and invertebrates to leaf litter decomposition in streams	5
1.2.2. Influence of litter quality and diversity on leaf decomposition and decomposers	6
1.2.3. Influence of decomposer diversity on leaf decomposition	8
1.3. Relationship between eutrophication and litter decomposition in streams	9
1.4. Objectives	11
<b>2. Materials and Methods</b>	
2.1. Study areas	13
2.2. Physical and chemical analyses of the stream water	14
2.3. Field experiment	15
2.4. Leaf bags processing and leaf mass loss	15
2.5. Leaf litter nutrient content	15
2.6. Macroinvertebrates from litter bags	16
2.7. Data analyses	16
<b>3. Results</b>	
3.1. Stream water characteristics	18
3.2. Leaf litter nutrient content	20
3.3. Leaf mass loss	21
3.3. Macroinvertebrates	26
<b>4. Discussion</b>	
4.1. Litter diversity effects	32
4.2. Eutrophication and litter diversity effects	34
<b>5. Conclusions</b>	37
<b>References</b>	38



## List of figures

<b>Figure 2.1.</b> Location of the sampling sites	14
<b>Figure 3.1.</b> Principal component analysis of the physical and chemical parameters of the stream water	20
<b>Figure 3.2.</b> Leaf mass loss of single species and mixtures of leaf species	22
<b>Figure 3.3.</b> Leaf mass loss for each level of litter species richness	23
<b>Figure 3.4.</b> Relationship between leaf mass loss and the eutrophication gradient	23
<b>Figure 3.5.</b> Net plant litter diversity effects on leaf mass loss	25
<b>Figure 3.6.</b> Relationship between leaf mass loss and leaf litter nitrogen content	25
<b>Figure 3.7.</b> Relationships between leaf mass loss and leaf litter C:N ratio	26
<b>Figure 3.8.</b> Invertebrate and shredder taxon richness and density	27
<b>Figure 3.9.</b> Net plant litter diversity effects on invertebrate taxon richness	28
<b>Figure 3.10.</b> Net plant litter diversity effects on shredder density	30
<b>Figure 3.11.</b> Relationships between PC1 scores and shredder density, shredder taxon richness and invertebrate taxon richness	31



## List of tables

<b>Table 3.1.</b> Physico-chemical characteristics of the stream water	19
<b>Table 3.2.</b> Nutrient content of single leaf litter type and leaf litter mixtures	21
<b>Table 3.3.</b> Summary of the nested ANOVA on leaf mass loss	24
<b>Table 3.4.</b> Summary of the nested ANOVAs on invertebrate and shredder density and taxon richness	29



## **1. Introduction**

### **1.1. Biodiversity and ecosystem functioning**

Over the past decade the debate on the relationships between biodiversity and ecosystem functioning (BEF) has emerged as a central issue in ecology (Duffy 2009). Concerns arose over the potential consequences of biodiversity loss due to anthropogenic activities to the functioning of ecosystems and implications to the services they provide to humanity (Duffy 2009; Lecerf and Richardson 2010; Loreau 2010).

Several hypotheses were proposed to explain the biodiversity-ecosystem functioning relationships. Some hypotheses are based on the assumption that several species are redundant (Naeem et al. 2002). This suggests that ecosystem functioning may not be affected by species loss as long as the remaining species can ensure the functions (Pascoal and Cássio 2008). The Rivet hypothesis (Ehrlich and Ehrlich 1981) states that loss of species does not affect ecosystem functioning until a threshold value beyond which ecosystem functioning becomes compromised (Johnsson et al. 1996; Naeem et al. 2002). Nevertheless, there may be species performing unique functions (keystone species) and its gain or loss may have significant effects on ecosystem functioning (Naeem et al. 2002). Another assumption is that the response of ecosystem functioning to species addition or loss does not follow a particular trend or pattern and therefore it is unpredictable or idiosyncratic (Naeem et al. 2002; Lawton 1994). According to the latter hypothesis, effects of species diversity may depend on the environmental context (Naeem et al. 2002). Moreover, species identity may have a great effect on ecosystem functioning and thus the sequence in which species are lost may differentially affect ecosystem functioning (Lawton 1994).

Results from meta-analyses have shown an overall positive relationship between assemblage diversity and the ecosystem function in which the assemblage participates (Balvanera et al. 2006; Cardinale et al. 2006; Schmid et al. 2009). Balvanera et al. (2006) measured biodiversity effects in single-studies as correlation coefficients of a given response variable; while Cardinale

et al. (2006) calculated the log ratio of responses to quantify diversity effects; finally, Schmid et al. (2009) aggregated data sets from the two former studies and analysed biodiversity effects through correlation coefficients, significances and signs. However, it is known that several factors can influence the responses of a given ecosystem process to biodiversity alterations (Balvanera et al. 2006; Schmid et al. 2009). Unfortunately, the vast majority of BEF experiments are rather simplistic and unless biological and environmental complexity is incorporated, results cannot provide realistic scenarios of biodiversity effects on ecosystems functioning (Hillebrand and Matthiessen 2009).

#### *1.1.1. Mechanisms behind BEF relationships*

Controversy has also focused on the mechanisms proposed to explain the relationship between biodiversity and ecosystem functioning (Hughes and Petchey 2001). Positive effects of species diversity on ecosystem functioning may occur because of complementarity effects in which communities will have better performances than that expected from individual species performance (Loreau et al. 2001). Differential capture of resources in space or time (niche partitioning or differentiation) and interactions between species that facilitate capture of resources among species or alleviate environmental adverse conditions (facilitation) lead to increased performance of more diverse communities (Cardinale et al. 2007; Hooper et al. 2005). Another type of mechanism responsible for positive effects of biodiversity on ecosystem functioning is the sampling effect. This mechanism assumes that more diverse communities will have higher probability of containing species that perform better than most species in the community (Hughes and Petchey 2001; Loreau and Hector 2001). However, this effect has been considered by some authors as an artifact of experiments that assumed communities as random assemblages of species from the total species pool (Hooper et al. 2005). As communities are not random assemblages of species and evidence points to differential contribution of individual species to ecosystem functioning, sampling effects must be considered when searching for mechanisms underlying BEF



relationships (Pascoal and Cássio 2008). A selection effect, rather than a sampling effect, was proposed by Loreau and Hector (2001) emphasizing the role of individual species traits in determining their performance in the presence of other species. They argued that complementarity and selection effects share the sampling process because a speciose community will always have higher probability of containing the best performing species either due to individual species traits or complementarity of species traits.

Complementarity and sampling (or selection) effects can occur simultaneously (Hooper et al. 2005; Loreau et al. 2001). A more diverse community may have higher diversity of traits which can lead to dominance of species with particular traits or to complementarity between species with different traits or even to dominance of a particular group of species or complementarity among certain groups of species (Loreau et al. 2001).

#### *1.1.2. Contribution of stream ecology to BEF research*

Studies in stream ecology have greatly contributed to advances in the debate on biodiversity-ecosystem functioning relationships. Aquatic ecosystems possess unique features that enable testing the hypotheses underlying biodiversity effects on ecosystem functioning (Giller et al. 2004). Studies in stream ecosystems have focused on the diversity effects of consumers (microbes and/or invertebrates) and resources (leaf litter) on organic matter decomposition (Kominoski et al. 2010; Lecerf and Richardson 2010; Srivastava et al. 2009). Experiments focusing on leaf litter diversity effects on decomposition showed outweigh of non-additive over additive effects, mostly negative (antagonistic) or both positive (synergistic) and negative effects (Rosemond et al. 2010). In a meta-analysis conducted by Srivastava et al. (2009), no clear pattern was observed regarding litter diversity effects on decomposition as the positive and negative effects across experiments cancelled each other. However, a recent meta-analysis conducted by Lecerf et al. (2011) concluded that synergistic effects were more frequent than antagonistic ones. Consumer diversity effects on decomposition appear to be strongly positive (Srivastava et al. 2009) due to the influence of microbial

consumers, particularly aquatic hyphomycetes (Bärlocher and Corkum 2003; Duarte et al. 2006) or invertebrate detritivores (Jonsson and Malmqvist 2003b). Facilitation and resource partitioning are the two mechanisms most likely responsible for positive diversity effects, although some negative effects may occur, particularly between invertebrates, due to competition (Gessner et al. 2010; Jonsson and Malmqvist 2003b).

## **1.2. Leaf litter decomposition in streams**

In low-order forested streams, the input of leaf litter from riparian vegetation is the major source of energy for aquatic food webs because shading and low water temperature limit primary production (Abelho 2001; Lecerf et al. 2007a). Leaf litter in-stream decomposition is a process that results in a variety of products such as inorganic compounds ( $\text{CO}_2$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ), several dissolved organic matter (DOM), fine particulate organic matter (FPOM) and biomass of decomposers, namely fungi, bacteria and invertebrate detritivores (Gessner et al. 1999; Hieber and Gessner 2002). The decomposition of leaf litter is a complex process that involves interplay of physical and biotic forces working simultaneously and being interdependent (Abelho 2001; Gessner et al. 1999). Leaching, considered the initial stage of leaf breakdown, may occur during the first 24h up to 7 days and is responsible for initial mass loss (up to 42%) mainly due to the loss of compounds such as tannins, polyphenols and soluble sugars (Abelho 2001; Gessner et al. 1999; Graça 2001). Colonization of leaf litter by aquatic microorganisms is known as conditioning; this process contributes to the degradation of litter material due to mechanical and enzymatic activity of microbes, and microbial growth that contributes to increases in leaf palatability for invertebrate shredders (Gessner et al. 1999; Graça 2001). Further decomposition of leaf litter occurs by physical and biotic fragmentation. Water flow is responsible by physical fragmentation through shear stress and abrasion of leaves (Gessner et al. 1999) and biotic fragmentation occurs due to feeding activity of invertebrate shredders and microbial activity (Abelho 2001; Gessner

et al. 1999; Graça 2001). The resulting FPOM is itself used by microorganisms and by invertebrate collectors, namely the collector-gatherers and collector-filterers (Graça 2001).

As litter decomposition is an integrative process it can be influenced by decomposer assemblages, stream water characteristics (Abelho 2001) and by leaf chemical and physical characteristics, such as nutrient and refractory compound contents (Lecerf and Chauvet 2008).

### *1.2.1. Contribution of microorganisms and invertebrates to leaf litter decomposition in streams*

Decomposer community is composed by microorganisms (bacteria and fungi) and invertebrate detritivores that all together participate in leaf litter decomposition. Several studies have suggested that fungi dominate microbial decomposing activity at early stages of litter decomposition (Baldy et al. 2007; Gulis and Suberkropp 2003a, 2003b; Pascoal and Cássio 2004; Pascoal et al. 2005a), while bacteria increases its relative contribution to litter decomposition only after plant litter has been partially broken down (Baldy et al. 2007). A recent global-wide experiment along a latitudinal gradient shows that microbial contribution to litter decomposition declines with latitude, whereas shredder contribution increases (Boyero et al. 2011). Studies on temperate streams have shown that invertebrate shredders are responsible for a greater portion of decomposition than microbes (Hieber and Gessner 2002; Kominoski et al. 2011; Lecerf et al. 2005). However, the role of shredders in litter decomposition may decrease in impacted streams. Microbes, especially fungi, accounted for a significant part of litter decomposition in eutrophic streams, when shredders were rare or absent (Baldy et al. 2007; Pascoal et al. 2005a).

Invertebrate shredders selectively feed on some leaf species due to microbial conditioning and leaf physical and chemical characteristics. Leaves with high nutrient content and low toughness can be easily consumed by shredders, whereas leaves with higher toughness are consumed by shredders only after a large period of microbial conditioning (Alonso et al. 2010; Sanpera-Calbet et al. 2009). Microbial conditioning can affect invertebrate feeding because microbes

through their enzymatic activities release compounds that invertebrates can more easily assimilate and because microbial biomass enhance the nutritional content of leaves for invertebrate consumption (Graça 2001). Furthermore, selective feeding of invertebrates has also been observed because leaves colonized by some fungal species are preferred over others, and preference also varies with the invertebrate species (Gessner et al. 2010).

### *1.2.2. Influence of litter quality and diversity on leaf decomposition and decomposers*

Litter decomposition is a critical ecosystem process influenced by the physical and chemical environment, decomposer communities and litter quality (Gartner and Cardon 2004; Hättenschwiler et al. 2005). LeRoy and Marks (2006) reported higher importance of leaf litter quality than stream water characteristics for litter decomposition rates, but differences in stream water parameters accounted for most variation in macroinvertebrate assemblages. Differences in decomposition rates between leaf species were primarily related to the physical and chemical characteristics of leaves (Benfield 2006). Chemical composition of leaf species can vary greatly, from nutrient-rich species, which are easily utilised by decomposers (labile), to species that are nutrient-poor or have high concentrations of compounds that are difficult to degrade (recalcitrant) or are even toxic to decomposers (Gessner et al. 2010; Hättenschwiler et al. 2005). Indeed, leaves with higher concentration of tannins and lignin and higher C:N ratio are more recalcitrant and have slower decomposition rates than more labile species, under similar environmental conditions (Hättenschwiler et al. 2005; Kominoski et al. 2007).

Under natural conditions, different riparian plant species grow closely together so the resulting decomposition of plant litter is the decomposition of the overall mixture of leaf species (Hoorens et al. 2003; Ostrofsky et al. 2007). By incubating leaf species in packs both alone and in mixtures of different leaf species, for a certain period of time, and then comparing litter mass loss (or remaining mass) in mixtures with that expected from single species, allows the detection of litter diversity effects on decomposition (Hui and Jackson 2008).

When decomposition of litter mixtures differs from the expected based on single species decomposition, it indicates non-additive diversity effects (Lecerf et al. 2007; Meier and Bowman 2010) which can be positive (faster than expected, i.e. synergistic) or negative (slower than expected, i.e. antagonistic) (Hoorens et al. 2003). An additive effect occurs when decomposition of leaf mixtures equals the expected one based on single-species decomposition (Hui and Jackson 2008).

Evidence of non-additive effects of leaf litter mixtures on decomposition has arisen from studies in terrestrial and freshwater ecosystems, and a clear tendency for synergistic effects was observed (Gartner and Cardon 2004; Hättenschwiler et al. 2005; Lecerf et al. 2011). Complex interactions between litter species with different litter chemistry can affect the decomposer activity and may be responsible for non-additive effects of litter mixtures on decomposition (Lecerf et al. 2011; Swan et al. 2009). Transfer of nutrients and other leaf compounds between leaf species can occur due to leaching and subsequent diffusion into the medium followed by assimilation by microorganisms colonizing the different litter species or due to microbial transportation of compounds within fungal hyphae that grows on different litter species (Gessner et al. 2010; Schindler and Gessner 2009). This is likely to increase decomposition of low-quality leaves and may contribute to synergistic diversity effects of litter mixtures. Antagonistic effects are also likely to arise when inhibitory compounds leached from recalcitrant species reduce decomposition of labile litter species (Schindler and Gessner 2009). Tannins can form complexes with fungal extracellular enzymes, inhibiting microbial growth and activity, while other polyphenols by complexing with proteins make nitrogen unavailable (Hoorens et al. 2003). Nevertheless, some authors claim that effects of litter mixtures are less likely to occur in streams where leached compounds are easily diluted and, thus, microbial assimilation is hampered (Gessner et al. 2010). Chemically and physically more diverse litter can benefit invertebrates due to changes in the relative abundance of nutrients that can be more appropriate to invertebrate needs and increase habitat complexity and stability (Lecerf et al. 2011). Invertebrate selective feeding may alter decomposition rates of low- and high-quality litter (Swan and Palmer 2006a,

2006b), although inter and intraspecific interactions, such as predation and competition may modulate feeding preferences (Lecerf et al. 2011).

### *1.2.3. Influence of decomposer diversity on leaf decomposition*

Increased diversity of decomposers appears to have positive effects on leaf litter decomposition (Srivastava et al. 2009). Results from manipulative experiments show that fungal diversity tends to have positive effects on leaf litter decomposition, although there is evidence of considerable redundancy among aquatic fungi (Pascoal and Cássio 2008). However, responses may vary depending on the environmental conditions (Fernandes et al. 2011; Pascoal et al. 2010). Synergistic interactions among fungi such as resource partitioning and facilitation are more likely to occur than antagonistic interactions (Gessner et al. 2010). Enzymatic activity can be complementary among fungi in terms of patterns of activity that can vary among fungal species, with environmental conditions and plant substrates. On the contrary, competition should be rare among fungi: the production and release of inhibitory compounds are not favoured due to water dilution (Gessner et al. 2010).

Invertebrate diversity also affects leaf litter decomposition. In a simple microcosm experiment Jonsson and Malmqvist (2000) used up to three detritivore species, alone and in mixtures, and observed that diversity increased decomposition. The authors suggested that facilitation and differences in the strength of interactions within or among species were responsible for such effects (Jonsson and Malmqvist 2003b). However, in a field enclosure experiment with up to three species of invertebrate detritivores, a reduction in leaf litter decomposition with increased detritivore diversity was found (McKie et al. 2009). In this case, inter-specific competition was stronger in more diverse species assemblages partially explaining the negative effect of detritivore diversity on decomposition (McKie et al. 2009). However, results differed with invertebrate density and environmental conditions (pH and nutrient availability).

It has been hypothesized that species traits and functional diversity rather than taxonomic diversity may better explain ecosystem functioning, but it seems that effects of richness versus compositional assemblages vary with the trophic

level (Lecerf and Richardson 2010). Leaf litter consumption by shredders has been positively related to fungal species richness, which in turn was positively related to leaf species richness pointing to an indirect effect of leaf species richness on leaf decomposition through trophic interactions (Lecerf et al. 2005). However, in litter mixtures with contrasting quality, overall macroinvertebrate abundance was low (Kominoski and Pringle 2009) despite high- and low-quality litter had stimulated or inhibited microbial biomass, respectively (Kominoski et al. 2007).

Species identity influences density-dependent effects on ecosystem functioning (Dangles and Malmqvist 2004; Lecerf and Richardson 2010; McKie et al. 2008); at the same level of species richness, less evenly distributed invertebrate assemblages (high species dominance) showed higher decomposition rates than assemblages more evenly distributed (low species dominance), but the maintenance of decomposition rates required more species in more even assemblages (Dangles and Malmqvist 2004). In addition, the effects of invertebrate diversity seem to be dependent on the environmental context (Gessner et al. 2010; Kominoski et al. 2010). Variability in diversity effects was observed seasonally (Dangles and Malmqvist 2004; Swan and Palmer 2004) and spatially (McKie et al. 2008) and can be related to abiotic factors and differences in community structure (Lecerf and Richardson 2010).

### **1.3. Relationship between eutrophication and litter decomposition in streams**

Freshwaters are among the most impacted ecosystems in the world and include the greater losses in biodiversity (Dudgeon et al. 2005) mostly due to anthropogenic activities (Weitjeirs et al. 2009). Eutrophication, from agricultural and industrial activities, urbanization and atmospheric deposition (Nijboer et al. 2004; Pascoal et al. 2005b), occurs when nutrient concentrations in the stream water increase (Smith et al. 1999). Inputs of nutrients in aquatic systems can alter nutrient cycles (Smith et al. 2006) and ecosystem processes, such as leaf

litter decomposition, through effects on biotic assemblages (Ferreira et al. 2006; Menéndez et al. 2011).

Generally, stimulation of litter decomposition occurs under increased concentration of nitrogen and/or phosphorus in the stream water, and it is frequently associated with increased activity of fungi and bacteria on decomposing leaves (Baldy et al. 2007; Chung and Suberkropp 2008; Ferreira et al. 2006; Gulis et al. 2006; Gulis and Suberkropp 2003a, 2003b; Menéndez et al. 2011; Pascoal et al. 2005a).

Several studies have shown that nutrient enrichment stimulates invertebrate biomass or density with positive effects on leaf litter decomposition (Chung and Suberkropp 2008; Greenwood et al. 2007; Gulis et al. 2006; Pascoal et al. 2003, 2005a; Rosemond et al. 2010). However, the in situ addition of moderate levels of nutrients affected microorganisms but not invertebrate assemblages (Ferreira et al. 2006). Alterations in invertebrate community structure are generally observed in streams impacted by eutrophication or other pollutants (Baldy et al. 2007; Lecerf et al. 2006; Pascoal et al. 2005a). Invertebrate shredders include several sensitive taxa that commonly decline under eutrophic conditions. Moreover, inorganic nitrogenous compounds such as ammonia, nitrates and nitrites can have toxic effects on aquatic biota (Camargo and Alonso 2006; Lecerf et al. 2006). Also, hypoxic or anoxic conditions that are usually associated with eutrophic and hypertrophic environments may lead to extensive kills of invertebrates and fishes (Camargo and Alonso 2006) and suppression of microbial activity (Pascoal and Cássio 2004).

Complex trophic interactions may influence the nutrient enrichment effects on leaf litter decomposition and decomposer organisms. Low-quality litter seems to respond more strongly than high-quality litter to nutrient enrichment in the stream water (Greenwood et al. 2007; Gulis et al. 2006; Rosemond et al. 2010) probably because nutrient limitation in low-quality litter leads to a faster nutrient uptake from the water by colonizing microbes increasing litter nutritional value (Cross et al. 2003; Gulis et al. 2006). It appears that fungi mediate positive responses of invertebrates to increased nutrient concentrations in the stream water due to an enhancement of litter nutritional value (Chung and Suberkropp 2008). Indeed, invertebrate abundance, biomass and secondary production



responds positively to the increase in litter quality and in microbial biomass and production (Chung and Suberkropp 2008; Cross et al. 2006). However, availability of resources over time might be reduced because of faster litter processing ultimately limiting some consumers (Greenwood et al. 2007). Long-term increases in water nutrient concentrations can potentially alter invertebrate community structure through a reduction of the assemblage evenness and increase in dominance of certain taxa (Davis et al. 2010).

#### **1.4. Objectives**

Plant litter decomposition is a key process in low-order forested streams that depends on the interactions between riparian vegetation, biotic communities and environmental factors. Freshwaters are among the most impacted ecosystems in the world mainly because of human activities. Increased nutrient loads and riparian vegetation loss or alteration can alter the functioning of stream ecosystems through negative impacts on aquatic biota. Macroinvertebrates are a diverse group of organisms that play different roles in litter decomposition. Invertebrate shredders are responsible for the degradation of large particles of organic matter and the vast majority of organisms in this group are very sensitive to stream water pollution. However, the interactive effects of eutrophication and riparian vegetation loss on litter decomposition and associated invertebrate assemblages are poorly understood.

In this study, we assessed the effects of riparian vegetation loss on leaf litter decomposition and associated invertebrate assemblages along an eutrophication gradient. Single-species and selected combinations of five riparian tree species (*Alnus glutinosa*, *Castanea sativa*, *Eucalyptus globulus*, *Platanus* sp. and *Quercus robur*) were incubated for 38 days in seven streams with increasing nutrient concentrations to assess if: 1) plant-litter decomposition and the associated invertebrates depended more on litter species diversity or quality, 2) leaf litter diversity effects on leaf mass loss and invertebrate communities can be predicted by comparing mass losses and invertebrate

density or diversity in mixed litter with those expected from the weighted sum of individual litter species effects, and 3) eutrophication alters the observed patterns. Leaf litter diversity effects on litter decomposition and associated invertebrate were expected to be positive because more diverse litter mixtures would provide more diverse resources and a more stable habitat. However, the quality of litter species (i.e., resources) was expected to have higher impact than litter species diversity. Finally, eutrophication was expected to alter the observed diversity effects because nutrients in the stream water can increase litter nutrient content influencing decomposer activity.

## 2. Materials and Methods

### 2.1. Study areas

Field experiment was conducted in seven streams of the Ave River basin located in the Northwest of Portugal (Fig. 2.1) in an area with different demographic density, agricultural and industrial activities (Pascoal et al. 2003). Seven streams, ranging from 2<sup>nd</sup> to 4<sup>th</sup> order, were chosen according to the nutrient levels of the stream water. Agra Stream is near the Ave River spring in Serra da Cabreira. Riparian vegetation is dominated by *Castanea sativa* Mill. and *Quercus* sp. and the stream substrate is composed mainly by boulders and pebbles. Three streams (Andorinhas, Oliveira and Agrela) are in mountain areas with some agricultural activity. The Andorinhas Stream presents a riparian vegetation corridor dominated by *Alnus glutinosa* (L.) Gaertn., *Quercus* sp. and *C. sativa* which provides a closed canopy, and the stream bed is composed by sand and gravel. At the Oliveira Stream, *A. glutinosa*, *Quercus* sp., *Platanus* sp. and *C. sativa* constitute the riparian vegetation, and boulders, pebbles and gravel constitute the substrate. Agrela Stream is bordered by *A. glutinosa*, *Quercus* sp. and *Eucalyptus globulus* Labill.; sand and silt dominate the substrate and some boulders are also present. The Selho River, the Costa Stream and the Couros Stream run through the city of Guimarães. The sampling site in the Costa Stream is in the city park; pebbles and gravel dominate the substrate, and the riparian vegetation is composed by *A. glutinosa*, *Quercus* sp., *Populus* sp. and *C. sativa*. The Selho River site is bordered by *A. glutinosa* and *Populus* sp. confined to a very small strip along the river; sand, gravel and boulders constitute the substrate. The Couros Stream site is bordered by agricultural fields and occasionally by *Populus* sp., and the stream bed is dominated by sand.



**Figure 2.1.** Location of the sampling sites in the Ave River basin, northwest of Portugal.

## 2.2. Physical and chemical analyses of the stream water

Physical and chemical parameters of the stream water were measured during the study period at each sampling site. Conductivity, pH, temperature 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 cold box (4°C) and used for chemical analyses within 24h. A HACH DR/2000 (Hach company, Loveland, CO, USA) photometer was used to quantify nitrate (HACH kit, method 8192), nitrite (HACH kit, method 8507), ammonia (HACH kit, method 8155) and reactive phosphorus (HACH kit, method 8048) concentrations, according to HACH manual.

### 2.3. Field experiment

Leaves of *A. glutinosa* (A), *C. sativa* (C), *E. globulus* (E), *Platanus* sp. (P) and *Quercus robur* (L.) (O) were collected just before abscission in autumn 2009, air-dried and stored until used. Groups of leaves of  $4 \pm 0.001$ g were placed in bags (30 x 23 cm) of 5-mm mesh. Combinations of species mixtures consisting of three levels of species richness were randomly selected in a total of 48 treatments as follows: 5 single-species, 3 combinations of 2 species (A+C, A+E, A+O), 3 combinations of 3 species (A+C+O, A+E+P, A+E+O) and all 5 species together (4 replicates per treatment). The total mass of species mixtures was equally partitioned and the exact mass of each leaf species was recorded. Each set of 48 leaf bags was immersed in each of the seven streams in a total of 336 bags. Stones were placed inside the bags to maintain them on the stream bottom. Leaf bags were immersed between the 10th and 12th November 2010. After 38 days leaf bags were retrieved, transported to the laboratory and processed within 24h.

### 2.4. Leaf bags processing and leaf mass loss

In the laboratory, leaf litter were removed from each bag and rinsed with tap water over nested sieves (250  $\mu$ m and 800  $\mu$ m) to remove sediments and collect macroinvertebrates. Leaf litter was freeze-dried to constant mass ( $72 \pm 24$ h) and weighed ( $\pm 0.0001$  g). Additional groups of approximately 4g of unexposed leaf species were freeze-dried to constant mass ( $72 \pm 24$ h) and weighed ( $\pm 0.0001$  g) to estimate initial dry mass.

### 2.5. Leaf litter nutrient content

Leaf material for nutrient analysis was ground to a fine powder and determination of carbon (C) and nitrogen (N) was done with a LECO-CNS 2000 elemental analyser in CACTI, Servicio de Análisis Instrumental, Universidade de Vigo (Spain). Results were expressed in %N, %C and C:N ratio of leaf litter dry mass.

## 2.6. Macroinvertebrates from litter bags

Macroinvertebrates retained on the battery of sieves were preserved in 96% ethanol, identified to the lowest possible taxonomic unit and assigned into two groups: shredders and non-shredders (Tachet et al. 2010).

## 2.7. Data analyses

Differences in stream water parameters between sites were assessed using one-way ANOVAs followed by Tukey's tests (Zar 1996). Conductivity, nitrates and ammonia data were ln-transformed to achieve normal distribution. When data did not follow a normal distribution, a non-parametric Kruskal-Wallis test was used.

Nested ANOVAs were used to test the effects of leaf species number, identity (nested in species number) and the level of eutrophication in the stream water (referred as stream) on leaf mass loss, invertebrate and shredder density and taxon richness. Leaf mass loss data was arcsine square root transformed and invertebrate and shredder density and richness data were  $\log(X+1)$  transformed when necessary to improve normality and alleviate heteroscedasticity of data.

Observed values of leaf mass loss in litter mixtures were compared with those expected from the sum of individual leaf species mass loss weighed by their contribution in the mixture (Fernandes et al. 2011; Pascoal et al. 2010). Differences between observed and expected leaf mass loss for each level of leaf species richness (net diversity effects) were tested against the null hypothesis that the average difference equalled zero (t-test). The same procedure was used to assess net diversity effects on invertebrate and shredder taxon richness and density.

A principal component analysis (PCA) was used to ordinate sites according to the stream water parameters, after standardization of the data (CANOCO version 4.5, Microcomputer Power, Ithaca, New York).

Relationships between leaf mass loss or invertebrate taxon richness and the eutrophication gradient defined by the scores obtained from the first PC axis

were assessed by non-linear regression. Linear regressions were used to assess relationships between the eutrophication gradient and shredders density and taxon richness. Relationships between leaf litter nutrient content (%N, %C, C:N ratio) and leaf mass loss for each stream were assessed by linear regression and slopes were compared by ANCOVA followed by Tukey's test.

Spearman rank correlations were used to examine the relationship between leaf litter nutrient content (%N, C:N ratio) and leaf mass loss and taxon richness or densities of invertebrates and shredders.

All statistical analyses were performed with STATISTICA 6 (StatSoft 2001).

### 3. Results

#### 3.1. Stream water characteristics

Analysis of the stream water parameters (Table 3.1.) showed that temperature was similar among sites. Conductivity ranged from 16 to 324  $\mu\text{S cm}^{-1}$  and was different between streams, except for Selho River and Costa Stream (one-way ANOVA, Tukey's test,  $p < 0.05$ ). Dissolved oxygen decreased and pH values increased from Agra Stream (11.2 and 5.3  $\text{mg L}^{-1}$ ) to Couros Stream (5.9 and 7.2  $\text{mg L}^{-1}$ ) (Kruskal-Wallis,  $p < 0.05$ ). The lowest concentrations of inorganic nutrients were registered in Agra Stream (Table 3.1). The Couros Stream had the highest concentrations of  $\text{N-NO}_2^-$ ,  $\text{P-PO}_4^{3-}$  (Kruskal-Wallis,  $p < 0.05$ ) and  $\text{N-NH}_4^+$  (one-way ANOVA, Tukey's test,  $p < 0.05$ ).  $\text{N-NO}_3^-$  concentration was highest in Agrela Stream, intermediate in Andorinhas Stream and lower in Oliveira and Agra streams (one-way ANOVA, Tukey's test,  $p < 0.05$ ).

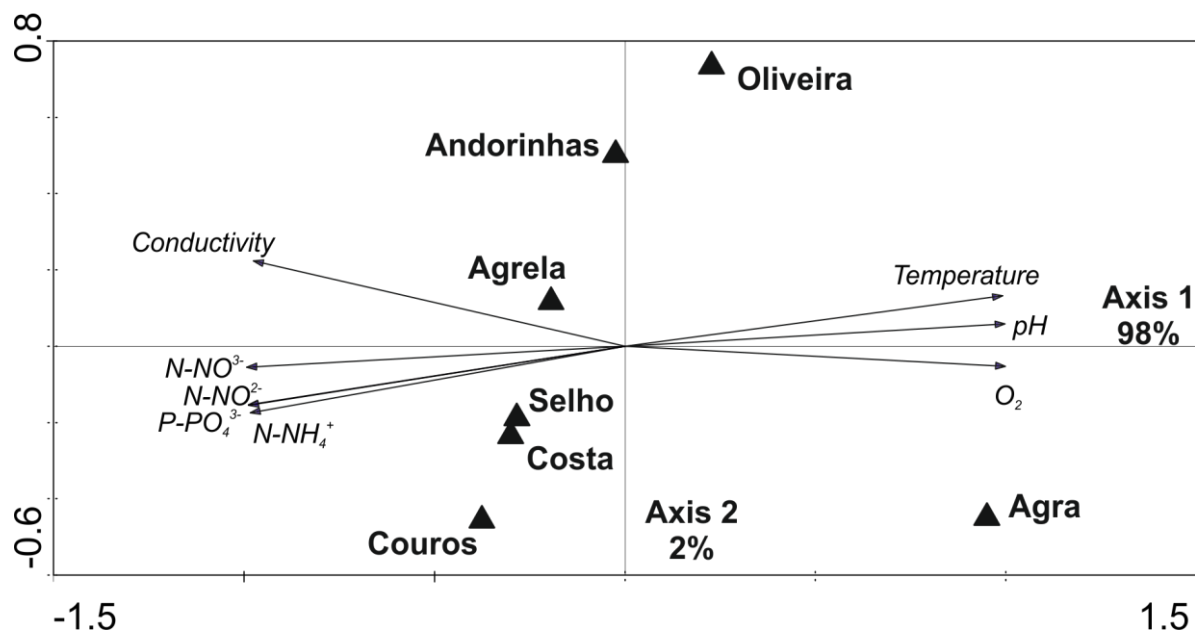
PCA ordination of the streams according to the physical and chemical parameters of the stream water (Fig. 3.1) showed that axis 1 and axis 2 explained 98% and 2% of the total variance, respectively. PCA ordinated the streams according to the eutrophication gradient defined by the first axis as follows: Agra Stream < Oliveira Stream < Andorinhas Stream < Agrela Stream < Selho River < Costa Stream < Couros Stream.



**Table 3.1.** Physico-chemical characteristics of the stream water in seven streams of the Ave River basin. Mean±SEM. n=3

Parameters	Agra Stream	Oliveira Stream	Andorinhas Stream	Agrela Stream	Costa Stream	Selho River	Couros Stream
Latitude N	41°36'35.24"	41°35'10.67"	41°34'11.24"	41°32'30.30"	41°26'53.77"	41°26'17.60"	41°26'14.93"
Longitude W	8°02'19.79"	8°13'30.46"	8°10'37.34"	8°19'10.20"	8°16'34.82"	8°19'21.22"	8°19'19.09"
Elevation (m)	776	232	210	269	218	149	149
Stream order	3	3	3	3	2	4	4
Temperature (°C)	8.9±1.32	11.6±1.26	12.1±1.22	12.7±0.62	13.9±1.10	12.9±0.65	14.6±1.13
pH	5.3±0.17	6.6±0.13	6.5±0.06	6.6±0.17	6.7±0.00	6.91±0.04	7.2±0.06
Conductivity (µS cm <sup>-1</sup> )	16±0.6	38.8±0.985	59±4.2	96.5±3.59	182±14.0	153.5±19.50	324±23.0
Oxygen (mg L <sup>-1</sup> )	11.2±0.16	11.1±0.29	10.2±0.24	10.3±0.10	9.8±0.19	9.95±0.110	5.9±0.19
P-PO <sub>4</sub> <sup>3-</sup> (mg L <sup>-1</sup> )	0.002±0.0011	0.004±0.0011	0.004±0.0011	0.007±0.0033	0.06*	0.004±0.0011	0.27±0.010
N-NO <sub>3</sub> <sup>-</sup> (mg L <sup>-1</sup> )	0.16±0.026	0.77±0.109	1.2±0.17	3.4±0.15	1.9*	3.0*	3.2*
N-NO <sub>2</sub> <sup>-</sup> (mg L <sup>-1</sup> )	0.005± 0.0010	0.005± 0.0010	0.006*	0.006± 0.0010	0.026± 0.0090	0.025± 0.0156	0.18± 0.006
N-NH <sub>4</sub> <sup>+</sup> (mg L <sup>-1</sup> )	0.01*	0.1*	0.010*	0.023±0.0132	0.31±0.110	0.3±0.16	3.7±0.65

\*, n=1



**Figure 3.1.** Principal component analysis (PCA) of the physical and chemical parameters of the stream water at the seven stream sites, Agra Stream, Oliveira Stream, Andorinhas Stream, Agrela Stream, Selho River, Costa Stream and Couros Stream. Directions of the arrows represent the maximum variation of each parameter.

### 3.2. Leaf litter nutrient content

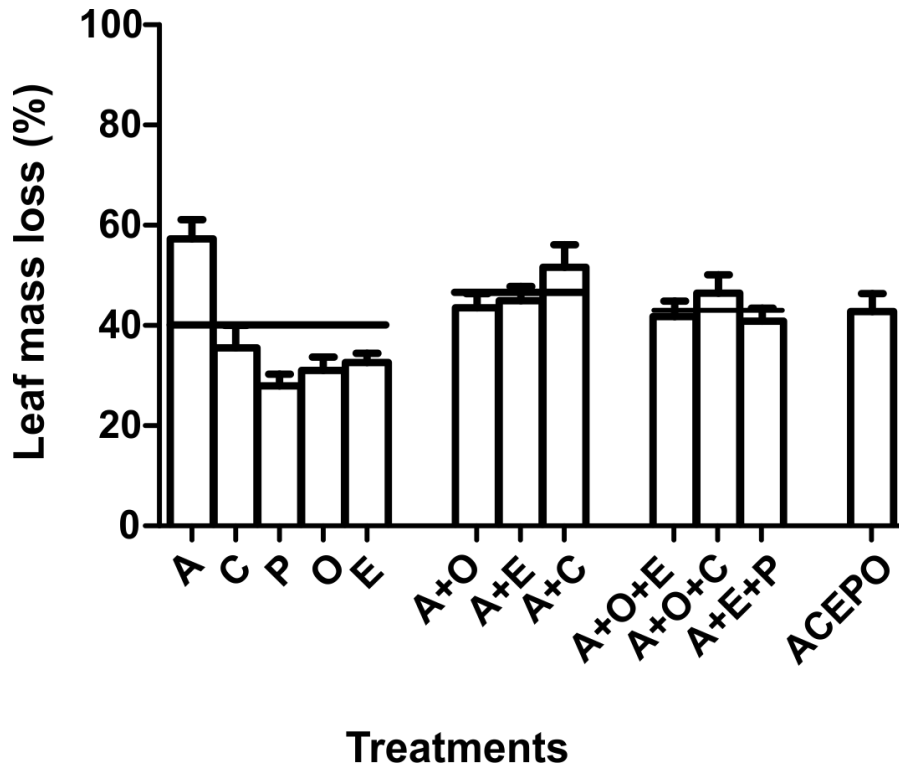
Nutrient content of leaf litter varied among litter single-species and mixtures (Table 3.2). In single-species treatments, nitrogen content increased from plane tree to oak, chestnut, eucalypt and alder leaves. Eucalypt leaves showed lower carbon content followed by chestnut, alder, oak and plane tree. The lowest C:N ratio was observed for alder which was followed by eucalypt, chestnut, oak and plane. Mixtures with combination of alder and chestnut (A+C) showed the highest nitrogen content and the lowest carbon content and C:N ratio. The combination with alder, eucalypt and plane tree leaves (A+E+P) had the lowest nitrogen content, intermediate carbon content, but the highest C:N ratio. The highest carbon content was observed for the combination with alder and eucalypt leaves (A+E).

**Table 3.2.** Nutrient content of single leaf litter type and leaf litter mixtures after 38 days of leaf immersion. M+SEM. n=7

	<b>N (% dry mass)</b>	<b>C (% dry mass)</b>	<b>C:N</b>
Alder (A)	4.8±0.11	46.4±1.53	9.7±0.25
Chestnut (C)	3.0±0.17	44.4±0.79	15.3±0.73
Oak (O)	2.7±0.15	47.2±0.58	17.7±0.89
Plane (P)	2.5±0.12	54.5±1.01	22.4±1.29
Eucalypt (E)	3.7±0.15	44.2±1.03	12.0±0.58
A+C	4.2±0.13	46.0±1.32	11.0±0.25
A+O	3.7±0.05	45.7±0.77	12.2±0.09
A+E	3.1±0.12	51.9±0.93	16.8±0.67
A+C+O	3.8±0.12	46.3±0.92	12.2±0.24
A+O+E	2.9±0.11	49.8±1.04	17.1±0.59
A+E+P	2.8±0.07	50.1±1.38	18.0±0.69
ACEPO	3.0±0.13	49.7±0.85	16.5±0.63

### 3.3. Leaf mass loss

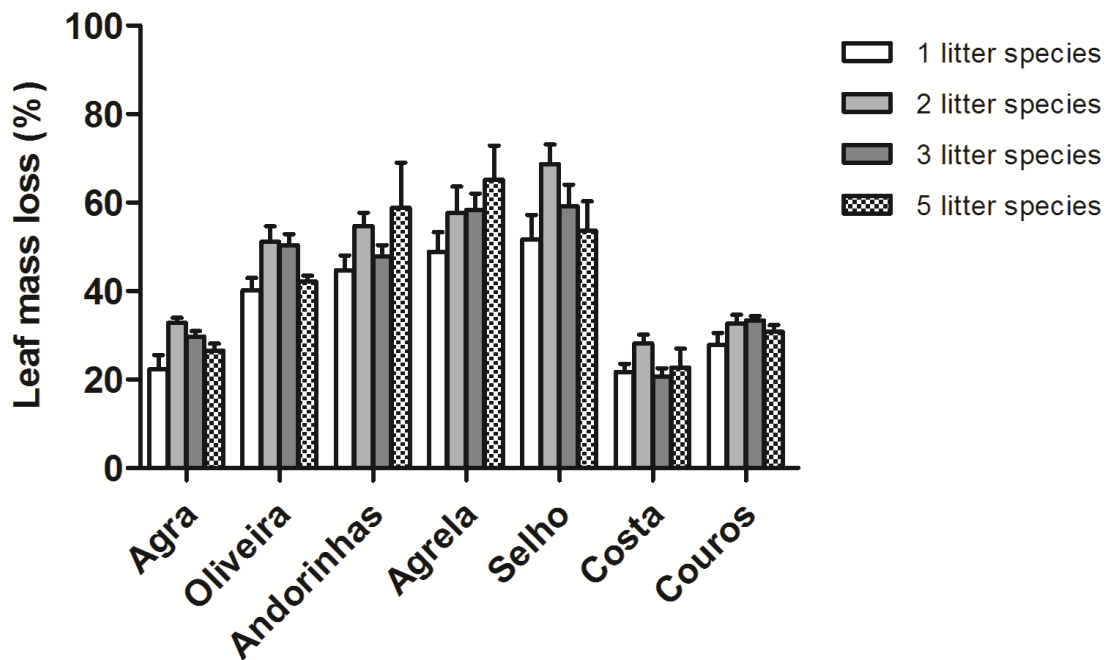
For single-plant species, leaf mass loss was higher for alder (A; 57.3%), followed by chestnut (C; 35.5%), eucalypt (E; 32.7%), oak (O; 31.0%) and plane tree (P; 28.0%). On average, leaf mass loss was higher in mixtures of 2 litter species with values ranging from 43.5% for A+O to 51.6% for A+C (Fig. 3.2). In treatments with mixtures of 3 litter species, mass loss was higher for A+O+C (46.4%) and lower for A+E+P (40.9%). For 5 litter species mixture, mass loss was 42.8%. Leaf mass loss varied among streams (Fig. 3.3) being highest in Agrela Stream and Selho River and lowest in the Costa Stream.



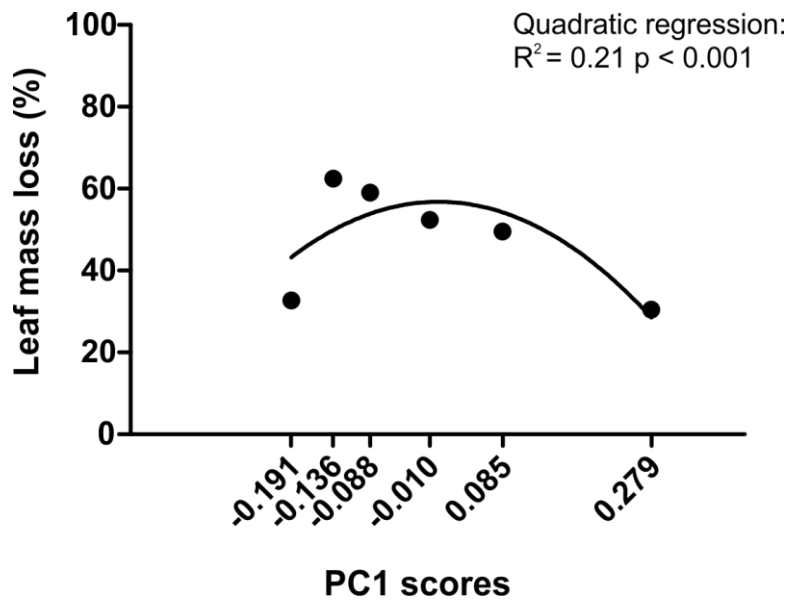
**Figure 3.2.** Leaf mass loss of single species and mixtures of leaf species after 38 days of immersion in all streams. *A. glutinosa* (A), *C. sativa* (C), *Platanus* sp. (P), *Q. robur* (O) and *E. globulus* (E).  $M \pm SEM$ . Horizontal lines indicate average values in treatments with one, two or three leaf species.

Number of leaf species and identity, stream, and the interaction between stream and species identity significantly affected leaf mass loss (three-way nested ANOVA,  $p < 0.05$ ; Table 3.3). Leaf mass loss of single species differed from those in mixtures but no difference was found between mixtures of 2, 3 and 5 litter species (Tukey's test,  $p < 0.05$ ).

A non-linear positive relationship was found between leaf mass loss and the eutrophication gradient ( $r^2 = 0.21$ ,  $p < 0.001$ ; Fig. 3.4). Higher decomposition occurred at intermediate levels of eutrophication, while lower or higher levels of eutrophication inhibited the process.



**Figure 3.3.** Leaf mass loss (%) for each level of litter species richness in seven streams of the Ave river basin. The streams were ordered according to the eutrophication gradient as defined by the first PC axis.



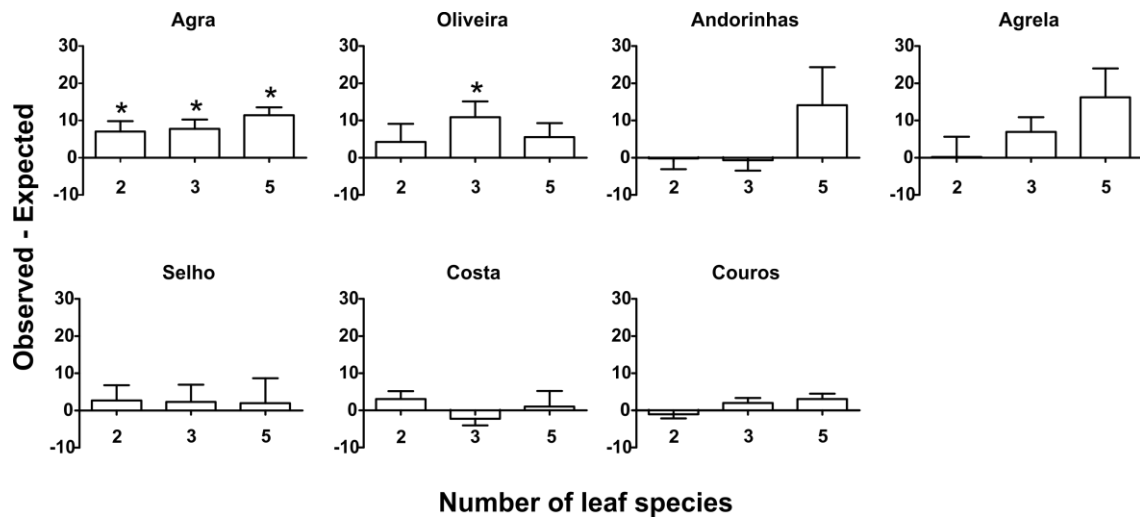
**Figure 3.4.** Relationship between leaf mass loss (%) and eutrophication gradient defined by the scores of the first PC axis. PC1 scores were: Agra Stream, 0.279; Oliveira Stream, 0.085; Andorinhas Stream, -0.010; Agrela Stream, -0.088; Selho River, -0.136 and Couros Stream, -0.191.

**Table 3.3.** Summary of the nested ANOVA of the effects of eutrophication level (referred as stream) and number of leaf species and identity (nested within number of species) on leaf mass loss.

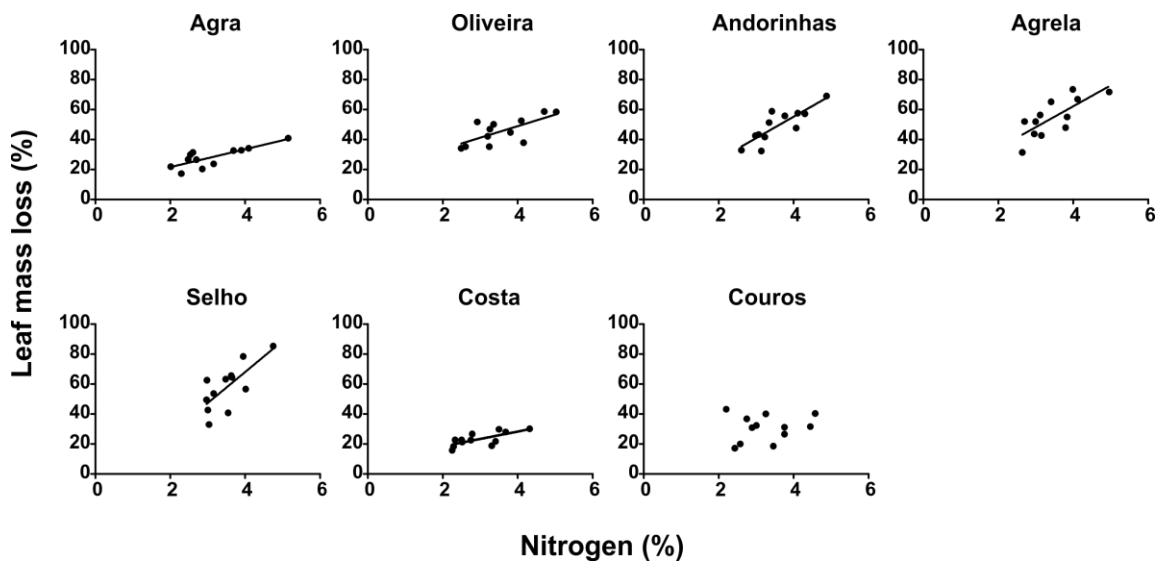
	<b>Source of variation</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>p</b>
Leaf mass loss	Stream	6	51708.9	8618.1	72.4	<b>&lt;0.001</b>
	Number of species	3	5459.6	1819.9	15.3	<b>&lt;0.001</b>
	Identity {Number of species}	8	16195.0	2024.4	17.0	<b>&lt;0.001</b>
	Stream * Number of species	18	2235.0	124.2	1.0	0.412
	Stream * Identity {Number of species}	48	9481.7	197.5	1.7	<b>0.007</b>
	Error	250	29767.5	119.1		

Effects of leaf litter diversity on leaf mass loss, assessed as the difference between the observed mass loss in mixtures and the expected values based on the weighed sum of individual mass losses, were positive for Agra Stream and Oliveira Stream, which showed the lowest levels of eutrophication (t-tests,  $p < 0.05$ ; Fig. 3.5). No significant differences were found between the observed and the expected leaf mass loss for the other streams (t-tests,  $p > 0.05$ ; Fig 3.5).

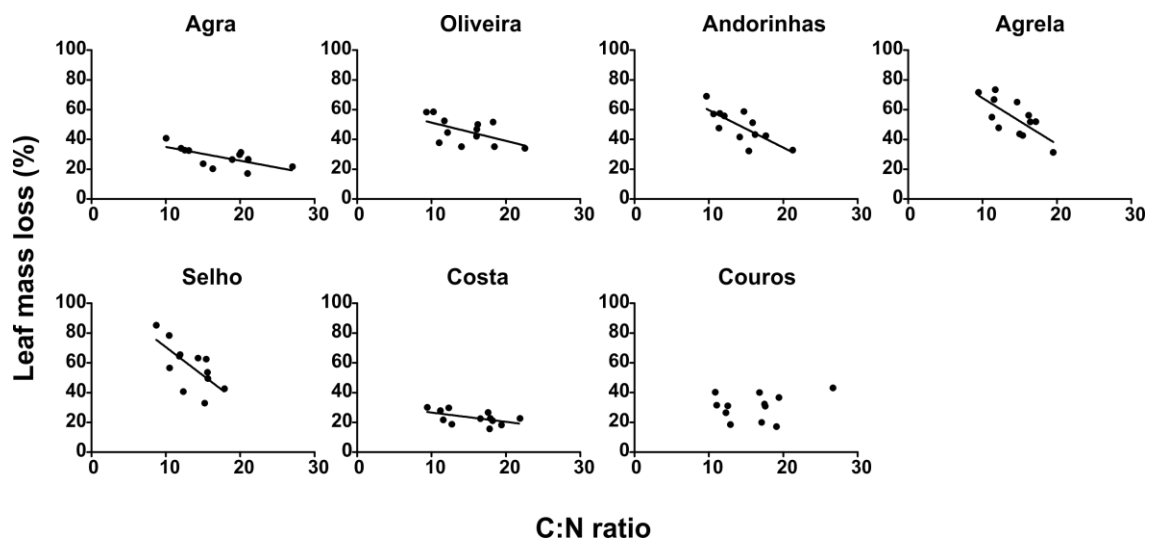
For all streams, except for Couros Stream, nitrogen content on leaves showed a positive relationship with leaf mass loss. The slopes of the regression between leaf mass loss and nitrogen content were steeper in streams with intermediate levels of eutrophication (ANCOVA,  $p < 0.001$ ; Fig. 3.6). A negative relationship was found between C:N ratio and leaf mass loss and the slopes of this relationship were also steeper at intermediate levels of eutrophication (ANCOVA,  $p < 0.001$ ; Fig. 3.7).



**Figure 3.5.** Net plant litter diversity effects on leaf mass loss in seven streams of the Ave river basin. Effects were estimated as the difference between observed leaf mass loss and expected based on the weighed sum of individual leaf species mass loss. \*, indicates significant differences from zero (t-tests,  $p < 0.05$ ).



**Figure 3.6.** Relationships between leaf mass loss (%) and leaf litter nitrogen content (%) for seven streams of the Ave river basin after 38 days of immersion. Agra Stream:  $r^2 = 0.53$ ,  $p < 0.001$ ; Oliveira Stream:  $r^2 = 0.25$ ,  $p < 0.001$ ; Andorinhas Stream:  $r^2 = 0.44$ ,  $p < 0.001$ ; Agrela Stream:  $r^2 = 0.26$ ,  $p < 0.001$ ; Selho River:  $r^2 = 0.27$ ,  $p < 0.001$ ; Costa Stream:  $r^2 = 0.17$ ,  $p < 0.01$ .



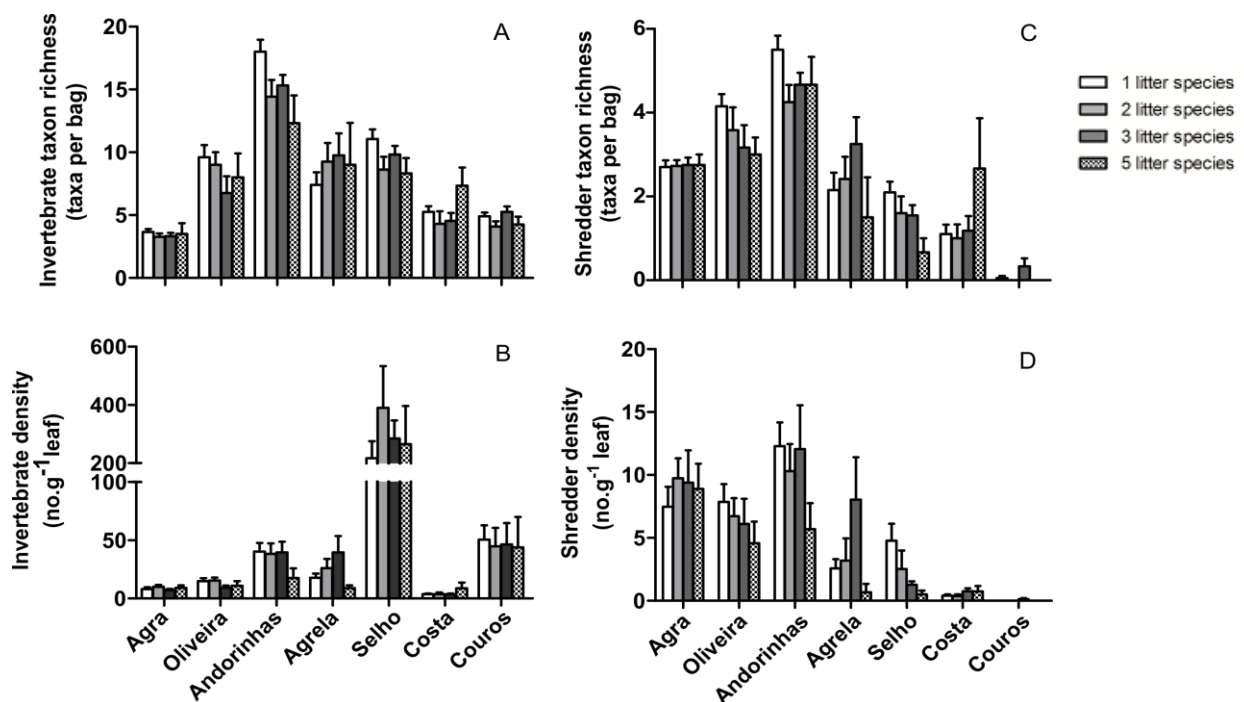
**Figure 3.7.** Relationships between leaf mass loss (%) and leaf litter C:N ratio in seven streams of the Ave river basin after 38 days of immersion. Agra Stream:  $r^2=0.37$ ,  $p<0.001$ ; Oliveira Stream:  $r^2=0.16$ ,  $p<0.01$ ; Andorinhas Stream:  $r^2=0.36$ ,  $p<0.001$ ; Agrela Stream:  $r^2=0.25$ ,  $p<0.001$ ; Selho River:  $r^2=0.24$ ,  $p<0.001$ ; Costa Stream:  $r^2=0.09$ ,  $p=0.036$ .

### 3.3. Macroinvertebrates

A total of 28612 organisms were recovered from litter bags comprising 74 invertebrate families. Nemouridae and Limnephilidae were dominant, followed by Asellidae in litter bags from Agra Stream. Dipteran families and especially Chironomidae were dominant in the other streams, except for Selho River where Oligochaeta became dominant. Trichoptera, as Limnephilidae, was very abundant in Oliveira Stream and Sericostomatidae was abundant in Andorinhas Stream. In Agrela Stream, Philopotamidae, Hydropsychidae were very abundant and Leuctridae and Oligochaeta were also well represented.

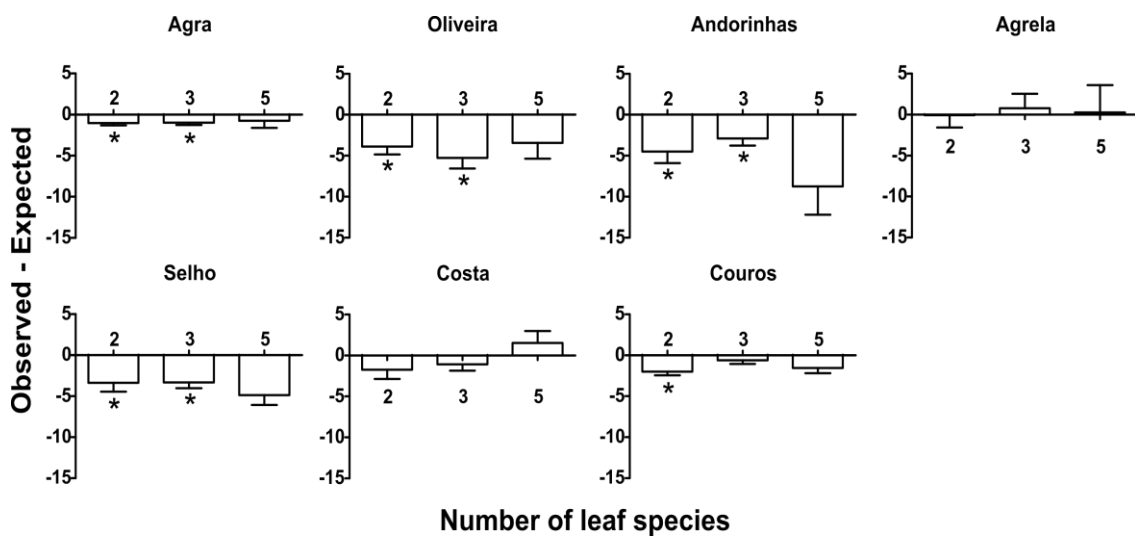


Invertebrate taxon richness varied between 4 taxa in Agra Stream and 16 taxa in Andorinhas Stream (Fig. 3.8A). Invertebrate density was highest in the Selho River (280 individuals.g<sup>-1</sup> leaf) and lowest in the Costa Stream (4 individuals.g<sup>-1</sup> leaf) (Fig. 3.8B). Stream and leaf species identity were the factors that significantly affected invertebrate density, while invertebrate taxon richness was affected by the stream and the number of leaf species (three-way nested ANOVAs,  $p < 0.05$ , Table 3.4).



**Figure 3.8.** Invertebrate taxon richness (A), invertebrate density (B), shredder taxon richness (C) and shredder density (D) on decomposing leaves in seven streams of the Ave river basin. M + SEM, n = 48.

Couros Stream had very low number of shredder taxa (<1 taxon per bag), while Andorinhas Stream showed the highest value (5 taxa) (Figure 3.8C). Shredder density ranged from 0.016 individuals.g<sup>-1</sup> leaf in the Couros Stream to 10 individuals.g<sup>-1</sup> leaf in the Andorinhas stream (Figure 3.8D). Stream and species identity significantly affected shredder density, while shredder taxon richness only varied with the stream (three-way nested ANOVAs, p<0.05; Table 3.4).



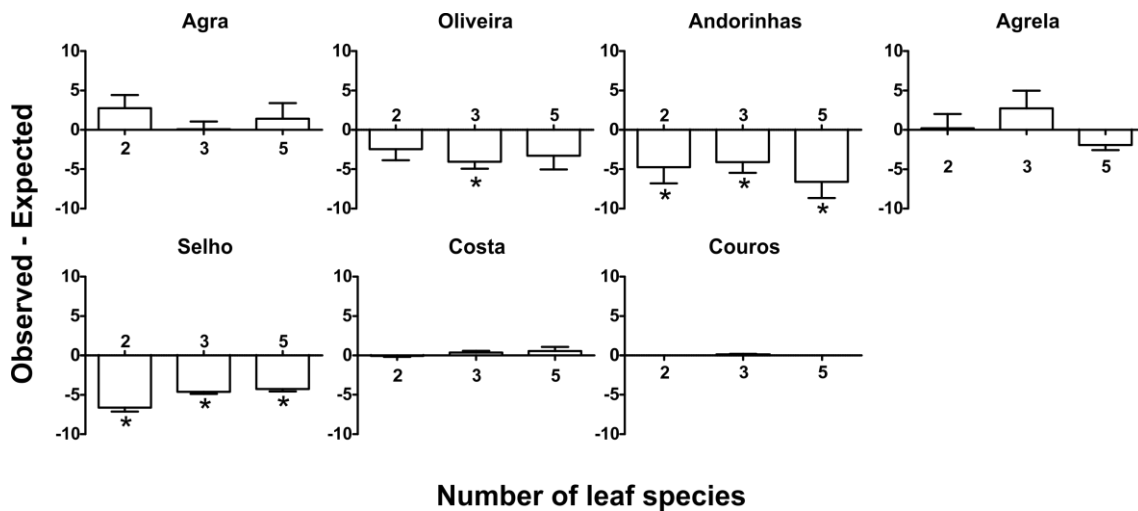
**Figure 3.9.** Net plant litter diversity effects on invertebrate taxon richness in seven streams of the Ave river basin. Effects were estimated as the difference between observed invertebrate taxon richness and that expected based on the weighed sum of invertebrate taxon richness on individual leaf species. \*, indicates significant differences from zero (t-tests, p<0.05).

**Table 3.4.** Summary of the nested ANOVAs of the effects of eutrophication level (expressed as stream) and number of species and identity (nested on number of species) on invertebrate and shredder density and taxon richness.

	Source of variation	df	SS	MS	F	p
Invertebrate density	Stream	6	58.49	9.75	55.34	<b>&lt;0.001</b>
	Number of species	3	0.15	0.05	0.28	0.841
	Identity {Number of species}	8	4.32	0.54	3.07	<b>0.003</b>
	Stream * Number of species	18	2.28	0.12	0.72	0.792
	Stream * Identity {Number of species}	48	5.23	0.11	0.62	0.976
	Error	241	42.45	0.18		
Invertebrate taxon richness	Stream	6	3683.1	613.9	42.6	<b>&lt;0.001</b>
	Number of species	3	302.4	100.8	7.0	<b>&lt;0.001</b>
	Identity {Number of species}	8	118.2	14.8	1.0	0.417
	Stream * Number of species	18	382.8	21.3	1.5	0.099
	Stream * Identity {Number of species}	48	397.6	8.3	0.6	0.988
	Error	243	3498.8	14.4		
Shredder density	Stream	6	33.82	5.64	66.76	<b>&lt;0.001</b>
	Number of species	3	0.25	0.08	0.99	0.396
	Identity {Number of species}	8	1.52	0.19	2.25	<b>0.025</b>
	Stream * Number of species	18	2.19	0.12	1.44	0.114
	Stream * Identity {Number of species}	48	3.72	0.08	0.92	0.631
	Error	241	21.11	0.08		
Shredder taxon richness	Stream	6	560.28	93.38	58.31	<b>&lt;0.001</b>
	Number of species	3	5.93	1.98	1.23	0.298
	Identity {Number of species}	8	18.01	2.25	1.41	0.195
	Stream*Number of species	18	43.58	2.42	1.51	0.086
	Stream*Identity {Number of species}	48	73.42	1.53	0.96	0.561
	Error	242	387.58	1.60		

Net leaf diversity effects on the taxon richness and density of invertebrates and shredders were mostly negative (i.e. antagonistic). Net effects of leaf diversity on invertebrate taxon richness were similar across streams (t-tests,  $p < 0.05$  in all streams, but not in Agrela and Costa streams; Fig. 3.9), and were only observed at lower levels of litter diversity. Significant antagonistic effects of litter diversity on shredder density were found in Oliveira and Andorinhas streams and in Selho River (Fig. 3.10).

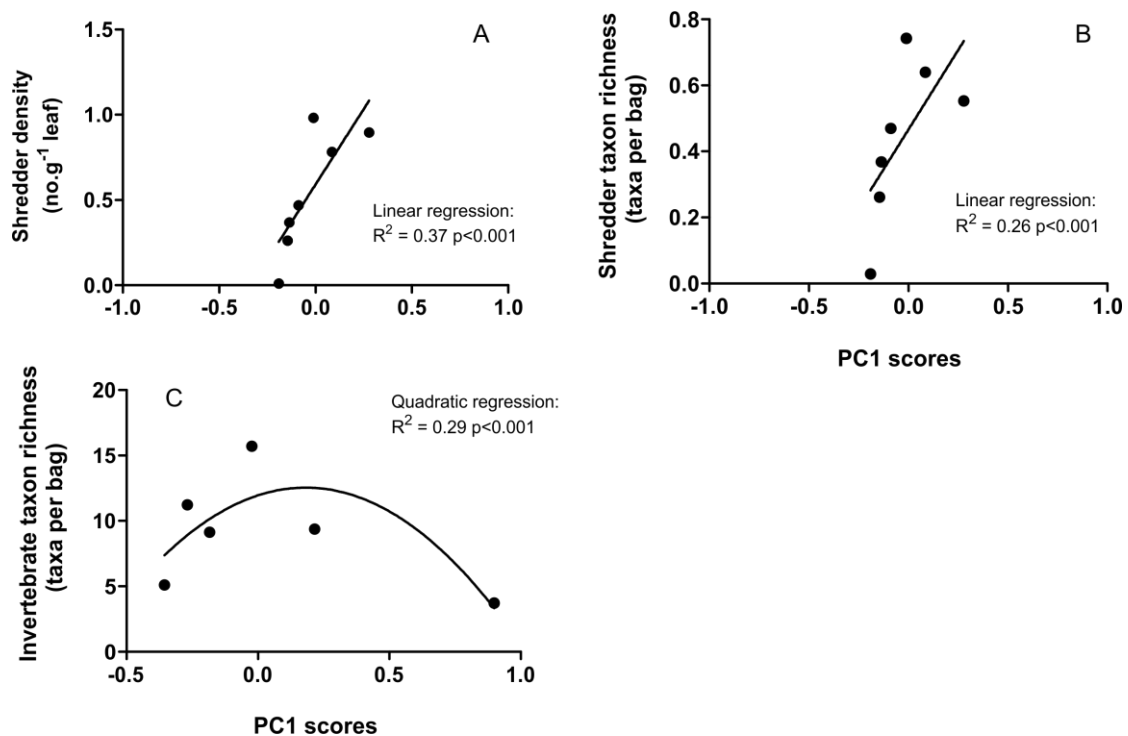
Significant positive correlations were found between leaf mass loss and invertebrate taxon richness ( $r = 0.44$ ,  $p < 0.05$ ), invertebrate density ( $r = 0.52$ ,  $p < 0.05$ ), shredder taxon richness ( $r = 0.33$ ,  $p < 0.05$ ) and shredder density ( $r = 0.34$ ,  $p < 0.05$ ). Number of leaf species correlated negatively with invertebrate taxon richness ( $r = -0.15$ ,  $p < 0.05$ ).



**Figure 3.10.** Net plant litter diversity effects on shredder density in seven streams of the Ave river basin. Effects were estimated as the difference between observed shredder density and that expected based on the weighed sum of shredder density on individual leaf species. \*, indicates significant differences from zero (t-tests,  $p < 0.05$ ).

Density and taxon richness of shredders and taxon richness of invertebrates were correlated with the eutrophication gradient. Increase in the eutrophication level led to a decrease in shredder density ( $r^2=0.37$ ,  $p<0.001$ ; Fig. 3.11A) and in shredder taxon richness ( $r^2=0.26$ ,  $p<0.001$ ; Fig. 3.11B). The relationship between invertebrate taxon richness and the eutrophication gradient was expressed by a non-linear quadratic regression ( $r^2=0.29$ ,  $p<0.001$ ; Fig. 3.11C), in which invertebrate taxon richness was highest in streams with intermediate levels of eutrophication.

Litter nitrogen content showed positive correlations with invertebrate taxon richness ( $r=0.26$ ,  $p<0.05$ ), invertebrate density ( $r=0.31$ ,  $p<0.05$ ), shredder taxon richness ( $r=0.14$ ,  $p<0.05$ ) and shredder density ( $r=0.19$ ,  $p<0.05$ ). C:N ratio showed negative correlations with invertebrate taxon richness ( $r= -0.28$ ,  $p<0.05$ ), invertebrate density ( $r= -0.29$ ,  $p<0.05$ ) and shredder density ( $r= -0.14$ ,  $p<0.05$ ). No correlation was found between shredder taxon richness and C:N ratio.



**Figure 3.11.** Relationships between PC1 scores and shredder density (A), shredder taxon richness (B) and invertebrate taxon richness (C).

## 4. Discussion

### 4.1. Litter diversity effects

Riparian vegetation diversity is known to affect leaf litter decomposition and the associated decomposer organisms. Effects of leaf litter diversity on litter decomposition are frequently positive (Gartner and Cardon 2004; Hättenschwiler et al. 2005; Lecerf et al. 2011), but litter species richness appears to have a weaker effect than the identity of species that constitute the mixture (Kominoski and Pringle 2009; Schindler and Gessner 2009; Swan and Palmer 2006a). Indeed, the presence of low- or high-quality litter in mixtures tends to slow or accelerate the decomposition process, respectively (Schindler and Gessner 2009; Swan et al 2008; Swan and Palmer 2004, 2006b; Taylor et al. 2007). In our study, leaf mass loss was affected by species number and to a greater extent by litter species identity. Leaf mass loss was highest in the combination of alder and chestnut, the most high-quality mixture (i.e. higher nitrogen content and lower C:N ratio). Alder is a nitrogen-rich leaf species (Taylor et al. 2007) and chestnut, despite its low nitrogen content, has a low cuticle with low thickness allowing faster microbial colonization and degradation (Canhoto and Graça 1996). This suggests that nutrient transfer may occur between litter types, probably mediated by fungi. If so litter nutritional value for invertebrate shredders might be enhanced contributing to faster leaf mass loss.

When alder leaves were mixed with leaf species of lower quality (i.e. low nitrogen content and high C:N ratio), such as oak, eucalypt or plane tree, leaf mass loss of mixtures consistently decreased. Indeed, leaves of oak and plane tree are considered slow decomposing litter species (Canhoto and Graça 1996; Gessner and Chauvet 1994; Sampaio et al. 2001). Plane tree leaves have high lignin and low polyphenolic contents, whereas oak leaves have more polyphenols but lower lignin content (Canhoto and Graça 1996; Gessner and Chauvet 1994; Schindler and Gessner 2009). Eucalypt leaves have high nitrogen content (Canhoto and Graça 1996; Sampaio et al. 2001) but also have a thick cuticle and oils, which makes colonization by decomposers difficult (Graça and Canhoto 2006). In our study, eucalypt leaves had intermediate

levels of nitrogen and C:N ratio and showed an intermediate leaf mass loss. Nitrogen tends to accumulate on litter during the decomposition process accompanied by a decrease of the litter C:N ratio (Manzoni et al. 2008). This may be associated with the nitrogen immobilization in the biomass of microbial decomposers (Ferreira et al. 2006; Gulis et al. 2006), and contribute to explain i) the positive correlations between litter nitrogen content and leaf mass loss and ii) the negative correlations between C:N ratio and leaf mass loss found in our study. The presence of low-quality leaves in the mixtures likely lowered leaf mass loss also because of leaching of oils and/or polyphenolic compounds which probably inhibited microbial and invertebrate consumption or due to an “armouring” effect of tougher litter species on more fragile litter protecting it against physical aggression (Swan et al. 2008) and preventing invertebrate access (Taylor et al. 2007).

A previous study showed that invertebrate assemblages are affected by leaf litter diversity and identity, as well as by stream physical and chemical characteristics (LeRoy and Marks 2006). This was confirmed in our study. Litter species identity affected invertebrate and shredder densities and litter species number affected invertebrate taxon richness. Surprisingly, shredder taxon richness was not affected by litter species number or identity. The diversity of invertebrate community shows an inconsistent response to resource diversity (Kominoski et al. 2010, 2011). This may be related to chemical and physical changes occurring in litter species throughout decomposition that alter the role of litter as source of food, habitat or refugia. Invertebrate colonization of plant litter does not occur at the same time and invertebrate resource use is conditioned by the availability of resources, invertebrate life history and competition (Kominoski et al. 2010; Kominoski and Pringle 2009).

In two (Agra and Oliveira) out of seven streams, the increase in litter species in the mixtures led to synergistic effects on litter mass loss, because higher mass losses were observed in mixtures than expected from the sum of mass losses of individual litter species. This suggests that factors other than the nutrient transfer between litter species and increased habitat stability, that favour decomposer activity, may have influenced leaf litter decomposition. The increase in litter species number in the mixtures led to antagonistic effects on

shredder density and taxon richness in Oliveira and Andorinhas streams and Selho River. In our study, litter mixtures with low-quality litter negatively affected density and taxon richness of shredders and total invertebrates. It is conceivable that high diverse litter mixtures containing low-quality litter will lead to a decrease in microbial conditioning with consequences on invertebrate assemblages due to longer persistence of low-quality litter in the mixtures. Monitoring of invertebrate colonization of litter mixtures during decomposition might help to clarify this question.

#### *4.2. Eutrophication and litter diversity effects*

In this study, concentrations of inorganic nutrients such as nitrites, nitrates, ammonia and phosphorus, in the stream water differed among the streams. Streams were ordinated according to an eutrophication gradient: Agra was the most oligotrophic stream, Oliveira and Andorinhas streams were moderately eutrophic, Agrela stream and Selho River were highly eutrophic, followed by Costa stream, and Couros stream was hypertrophic. Leaf mass loss was highest at moderate and moderately high levels of eutrophication than at oligotrophic or hypertrophic streams. These results are consistent with i) a nutrient enrichment experiment in which higher leaf decomposition was found in a stream with moderate nutrient concentrations (Chung and Suberkropp 2008), ii) studies that observed higher leaf decomposition with increased levels of eutrophication (Duarte et al. 2009; Menéndez et al. 2010; Pascoal et al. 2001, 2003; Pascoal and Cássio 2004), and iii) inhibition of decomposition at elevated eutrophication (Lecerf et al. 2006; Pascoal et al. 2005a). The positive correlations between leaf mass loss and leaf nitrogen content were stronger in streams with moderate and high levels of eutrophication. The increase in nutrient concentration in the stream waters often leads to an increase in fungal biomass (Chung and Suberkropp 2008; Ferreira et al. 2006; Gulis et al. 2006; Gulis and Suberkropp 2003a, 2003b; Pascoal and Cássio 2004). Microbes can uptake nutrients (e.g. nitrogen) from leaves and also from the water column, thus increasing nutrient content in decomposing leaves (Cross et al. 2003; Menéndez et al. 2011) and lowering the C:N ratios.



Invertebrate taxon richness increased in streams with moderate eutrophication (Gulis et al. 2006), but decreased in highly-polluted streams (Pascoal et al. 2003). Also, in our study, invertebrate taxon richness was higher at moderate and high levels of eutrophication, but was lower at oligotrophic and hypertrophic streams. This pattern was similar to that observed for litter decomposition, supporting that invertebrates contributed significantly to the decomposition process as found by others (Lecerf et al. 2006; Pascoal et al. 2005a). Density of tolerant invertebrates responds positively to eutrophication (Chung and Suberkropp 2008; Pascoal et al. 2003). However, in hypertrophic streams, invertebrates can decrease due to the presence of toxic compounds, such as ammonium and nitrite, with negative effects to decomposing of leaf litter (Lecerf et al. 2006). In our study, shredder taxon richness and density consistently decreased along the eutrophication gradient and shredders were practically absent in Costa Stream. In addition, eutrophication led to shifts in macroinvertebrate communities: Nemouridae, Limnephilidae and Asellidae dominated the litter bags in Agra stream; Chironomidae became dominant in Oliveira, Andorinhas, Agrela and Couros streams, while Oligochaeta dominated the invertebrate assemblage in Selho River.

Litter diversity effects on mass loss were suppressed along the eutrophication gradient established in our study. This is consistent with the findings of Rosemond et al. (2010) who observed suppression of litter diversity effects in a nutrient enriched stream. They suggested that high nutrient levels in the stream water can increase litter nutrient content via microbial uptake leading to a homogenization of nutrient content in litter mixtures and allowing detritivore unselective feeding. On the other hand, more diverse litter mixtures can translate in more heterogeneous habitat which might support more diverse consumer communities (Kominoski and Pringle 2009). Niche overlapping is greater within species than between species and hence competition is more likely to occur within species (McKie et al. 2009). It is possible that a homogenization of litter nutrient content in litter mixtures might result in higher competition between species leading to the negative effects of litter diversity on invertebrate community in the streams with intermediate and high levels of eutrophication. As it was already mentioned, shredder taxon richness and

density decreased along the eutrophication gradient. It is possible that an inhibition of microbial conditioning due to harmful effects of eutrophication could have occurred. Although we can assume that microbial activity on leaves had occurred, as litter nitrogen content had a positive relationship with leaf mass loss, it is possible that other factors such as the harmful effects of certain nitrogen compounds (e.g. ammonia) in the stream water may have influenced macroinvertebrate assemblages resulting in i) the loss of diversity effects on leaf mass loss and ii) negative effect of litter diversity on invertebrates assemblages.

## 5. Conclusions

Overall, our study supported that leaf litter diversity effects differed for litter decomposition and invertebrate assemblages. The diversity loss of riparian vegetation can have an impact on litter decomposition, but plant litter composition might have a greater role in controlling the decomposition process and associated biotic assemblages. We also found that effects of nutrient levels in the stream water can overwhelm effects of litter mixtures. These results clearly illustrate the great impact that anthropogenic activities can have on dynamics of litter processing in streams and on the biotic assemblages that participate in this process. The increase of nutrient concentration in the stream water seemed to suppress leaf litter diversity effects on litter decomposition, while the effects on invertebrates, particularly on shredders, were clearly negative. In our study, nitrogenous compound concentrations in the stream water were much higher than generally found in most studies and it is possible that toxic effects of nitrogenous compounds, such as ammonia, on invertebrates were captured. However, further studies are needed to fully understand the interactions between riparian vegetation loss, stream water eutrophication and the mechanisms that drive these interactions.

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