UNIVERSITAT DE BARCELONA

The role of leaf litter quality and streambed hydro-morphology on in-stream leaf litter dynamics

El rol de la qualitat de la fullaraca i de la hidro-morfologia del tram fluvial en les dinàmiques de la fullaraca als rierols

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Programa de doctorat en Ecologia, Ciències Ambientals i Fisiologia Vegetal

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Memòria presentada per el Sr. Elliot Bastias Álamo per optar al grau de doctor per la Universitat de Barcelona

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A mi abuelo,

y a toda la familia

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Leaf litter dynamics in stream ecosystems

Leaf litter inputs supply to streams organic carbon (C) and nutrients and thus, these inputs constitute relevant organic matter subsidies to the recipient streams (Likens and Bormann 1974; Wallace et al. 1999; Larsen et al. 2016). Once leaf litter enters the streams it can be retained and spatially distributed within the stream channel or be transported downstream. Once retained, leaf litter inputs can be processed by the recipient streams through the release of soluble compounds from the leaf litter (i.e., leaching process), the microbial decomposition of leaf litter constituents (i.e., leaf litter mineralization) and the physical fragmentation of leaf litter mediated by water abrasion and macroinvertebrate activity. Therefore, understanding the dynamics of leaf litter inputs in streams involves assessment of retention, spatial distribution and processing of leaf litter inputs (Figure 1.1.). Furthermore, leaf litter processing in streams is not an isolated process because it can be linked with the dynamics of solutes of these streams. In fact, leachates from leaf litter provide to the streams dissolved organic matter (DOM), as well as, dissolved nutrients (i.e., nitrogen [N] and phosphorous [P]). Also, the mineralization of leaf litter inputs implies the releasing of dissolved forms of C and nutrients to the stream water column (Webster et al. 2000; Webster et al. 2009). In addition, microbial communities inhabiting leaf litter uptake dissolved forms from water column during leaf litter decomposition (Kaushik and Hynes 1971).

Leaf litter dynamics in streams has been mostly studied by determining the controlling factors of leaf litter processing, ignoring if leaf litter inputs are retained within the streams, and how these inputs are spatially distributed within the streambed (Woodward et al. 2012). In addition, despite the obvious linkage between leaf litter processing and in-stream solute dynamics, few studies provided information about the main drivers

controlling this connection. Therefore, in order to understand how leaf litter dynamics operates in stream ecosystems (Figure 1.1.), the present thesis aims to examine (a) how the hydro-morphological characteristics of the recipient stream can influence the retention, spatial distribution, physical fragmentation and decomposition of leaf litter inputs. Moreover, we also examine the interaction between leaf litter processing and in-stream dynamics of dissolved organic carbon and dissolved inorganic nitrogen and how it varies depending on the leaf litter species considered.

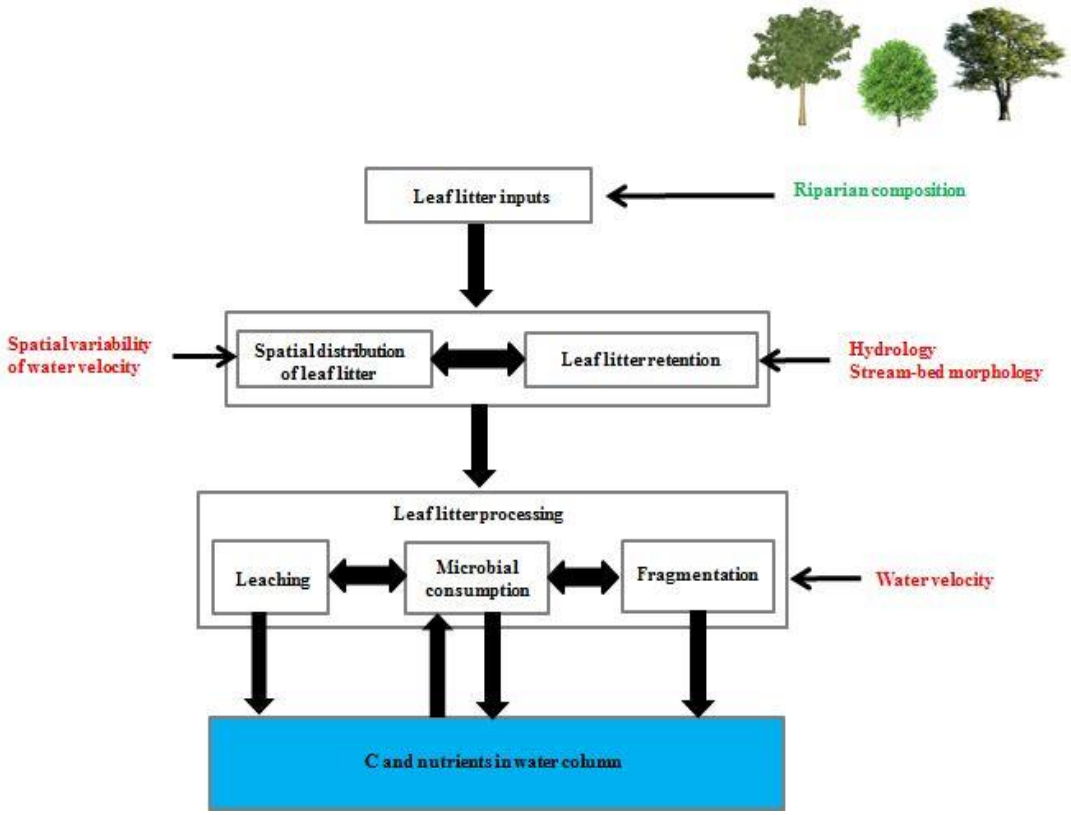

Figure 1.1. The figure describes the leaf litter dynamics in streams which includes: leaf litter inputs, the retention and spatial distribution of leaf litter within the stream and the leaf litter processing, which can be constituted by the leaching process, the microbial consumption of leaf litter constituents and the leaf litter fragmentation. We show how the riparian composition (green) and the hydro-morphological characteristics of the recipient stream (red) can influence the dynamics of leaf litter inputs in streams.

1.2. The influence of the composition of riparian vegetation on leaf litter inputs to streams

The composition of the riparian forest has important implications for the dynamics of inputs of leaf litter to streams because the riparian forest may ultimately dictate the quality of leaf litter inputs to streams. Quality of leaf litter is commonly assessed by its elemental composition (i.e., the content of C, N and P), and the relative proportion among these elements (Melillo et al. 2001). In general, leaf litter with high N and P content relative to C content is more easily processed by microbial decomposers (i.e., fast decomposition) than leaf litter with low relative content of N and P (Webster and Benfield 1986; Enriquez et al. 1993). For example, decomposition of alder (*Alnus glutinosa*) species is usually faster than that for other leaf litter species such as black poplar (*Populus nigra*) or sycamore (*Platanus X hispanica*) (Webster and Benfield 1986) because, the elemental C:N ratio of alder is lower relative to these species. Regardless of the C:N ratio, other leaf litter species such as black locust (*Robinia pseudoacacia*) and sycamore contain a high proportion of C-recalcitrant compounds such as lignin or tannins; and thus, these species constitute a source of low-quality substrate for stream microbial decomposers (Webster and Benfield 1986). In fact, litter quality is also related to the complexity of organic C molecules that constitute leaf litter (Webster and Benfield 1986). Simple organic compounds in leaf litter, such as soluble polysaccharides, are labile C sources; and thus, are easily degraded and consumed by microbes. In contrast, more complex C compounds in leaf litter are recalcitrant C resources; and thus, are more costly to be used by microbes (Sinsabaugh et al. 1993). Relatively higher proportions of recalcitrant C compounds in leaf litter have been negatively related to the leaf litter decomposition (Gessner and Chauvet 1994; Schindler and Gessner 2009). Overall this information indicates that the species composition of

the riparian forest, which can be influenced by the climatic setting of the region, can dictate the quality of leaf litter inputs to streams; and thus, ultimately influence how these inputs can be used by stream microbial assemblages.

In Mediterranean regions, vegetation is adapted to water stress; and thus, it is typically sclerophyllous and ever-green (Bunn 1986; King et al. 1987; Britton 1990). In the riparian zones of these regions, deciduous woody species can usually develop because adjacent streams provide optimal hydrologic conditions to riparian species that relay on water availability from phreatic level in the forest soil (Shmida 1981; Holstein 1984). In sub-humid Mediterranean areas, riparian forest is usually dominated by alders. Other riparian tree species such as black poplar, ash (*Fraxinus excelsior*) and sycamore can be present. In addition, currently black locust, an invasive tree species, is also common in riparian forests of these regions. The relative dominance of these species in the riparian forest depends on the degree of aridity of the forest soils (Maanri et al. 1994; Fisher SG 1995). Under increasing degree of aridity, deciduous tree species such as alder and black poplar are more restricted to grow near the stream channel. In contrast, species such as black locust are more adapted to grow under dry conditions (Maamri et al. 1994) and thus, can develop further away from stream channel. Thus, composition of the tree species in riparian forest of Mediterranean regions is subjected to the hydrological linkage between the stream and the riparian zone (Maanri et al. 1994).

Species composition of riparian forest can also influence the temporal pattern of leaf litter inputs to streams. The inputs of leaf litter from riparian zones to streams mostly occur during autumn. However, some studies have found that the temporal pattern of leaf litter inputs can vary among years due to the high inter-annual variation of weather

conditions. In wet years, leaf litter inputs are concentrated in short-term pulses during autumn; while in dry years, leaf litter inputs can occur over extended periods from mid-summer to end of autumn (Molinero and Pozo 2004; Acuña et al. 2007). However, in Mediterranean regions, leaf litter inputs can also be significant during summer due to hydric stress conditions on riparian tree species (Acuña et al. 2007). Although the leaf litter inputs to streams have been well described, previous studies considered leaf litter inputs as a bulk of species, ignoring the relative importance of different riparian tree species providing leaf litter into the streams.

1.3. Retention and spatial distribution of leaf litter inputs in streams

The amount of leaf litter inputs available to stream communities (i.e., leaf litter standing stocks) is not only influenced by the leaf litter inputs from riparian forest, but also by the probability of these inputs to be retained within the stream (i.e., retentiveness). In fact, once leaf litter enters the streams it can either be retained within the stream channel or be transported downstream depending on the hydrological conditions and the morphology of the stream channels (Fisher and Likens 1973, Larrañaga et al. 2003, Cordova et al. 2008). Previous studies have shown that the stream retentiveness for leaf litter decreases as discharge increases (Snaddon et al. 1992, Raikow et al. 1995, Dewson et al. 2007). Under high discharge the accumulation of particulate organic matter in the stream channel is dislodged, kept in suspension by turbulence, and transported to longer distances (Fisher and Likens 1973, Larrañaga et al. 2003, Cordova et al. 2008). In contrast, under low discharge, stream retentiveness tends to be high due to the high interaction between the particles and the streambed substrata (Speaker et al. 1984; Lamberti et al. 1989; Mathooko et al. 2001). Under these conditions, leaf litter tends to

buildup in the streambed at locations where shear stress is sub-critical (e.g. pools) or where leaves become trapped by obstacles such as wood, cobbles or boulders (Larrañaga et al. 2003, Cordova et al. 2008). Once retained, leaf litter inputs can be re-suspended whenever discharge conditions increase (Webster et al. 1994; Wallace et al. 1995). Thus, leaf litter retention is not a static, but a dynamics process following the hydrologic regimes of the recipient streams. Nevertheless, under baseflow conditions leaf litter retention has been assumed as static process, because soon after leaf litter enters into the stream its spatial distribution becomes stable. However, there is no empirical evidence of this fact and thus, information of how leaf litter retention in streams operates under baseflow conditions is still lacking.

1.4. Processing of leaf litter inputs in streams

Once leaf litter falls into the stream, it becomes processed through different in-stream mechanisms, assuming that it gets retained in the stream channel. In-stream leaf litter processing usually comprises 3 phases: (a) an initial rapid loss of matter due to leaching of dissolved constituents, (b) a successive microbial development on leaf litter surface which drives decomposition of leaf litter, and (c) the fragmentation of leaf litter due to physical factors and the activity of macroinvertebrate using leaf litter as a food source (Webster and Benfield 1986).

During a short period after its input into the stream (i.e., ~24h), leaf litter loses soluble organic and inorganic compounds (i.e., leachates) to the water column (Webster and Benfield 1986; Wymore et al. 2015). Leaf litter retained in the stream channel is also rapidly colonized by microbes (fungi and bacteria), which are the main biotic agents

involved in leaf litter decomposition (Kaushik and Hynes 1971). The main mechanism of fungi and bacteria to decompose leaf litter tissues is the production of extracellular enzymes, which can degrade high-molecular-weight compounds from leaf litter into low-molecular-weight compounds (Romaní et al. 2012, 2016). These low-molecular-weight compounds can then be assimilated by microbial communities (Rogers 1961). Therefore, the use of leaf litter as energy and matter resources to microbial assemblages (i.e., leaf litter decomposition) depends on the specific exoenzymatic activity associated to these microbial organisms (Slater and Lovatt 1984). In-streams, leaf litter decomposition is influenced by both internal factors of the leaf litter (i.e. chemical and physical characteristics of the leaves) and external environmental factors (i.e., stream characteristics). On the one hand, the internal factors of the leaf litter, which potentially influence its decomposition in streams can be divided into three categories: (a) content of essential elements in the leaf tissue; (b) fiber content; and (c) presence of chemical inhibitors (Webster and Benfield 1986). On the other hand, environmental factors such as water temperature (Ferreira and Chauvet 2011), dissolved nutrient concentrations (Ferreira and Chauvet 2011; Woodward et al. 2012), dissolved oxygen and water pH, among others, can influence leaf litter decomposition among streams (Webster and Benfield 1986). Leaf litter inputs are also subjected to mechanical fragmentation during their decomposition. In this regard, there are some evidences that water velocity of the stream habitats where leaf litter retains is one of the main factors explaining the physical fragmentation of leaf litter inputs in streams (Witkamp and Frank 1969, Hodkinsonm 1975; Ferreira and Graça 2006). Water velocity can be patchily distributed within a stream as a result of the interaction between stream flow and streambed morphology. However, despite the high variability of water velocity within the stream channel, the role of water velocity on in-stream leaf litter processing has not been addressed yet.

Furthermore, the physical fragmentation of leaf litter inputs is also mediated by the activity of invertebrates shredders (e.g., McDiffett 1970 Cummins 1974; Anderson and Sedell 1979), which increases the rate at which leaf litter is converted to fine particles (Wallace et al. 1982; Cuffney et al. 1990) and dissolved organic matter (DOM; Meyer and O'Hop 1983).

1.5. The influence of leaf litter inputs on in-stream dynamics of dissolved C and N

After seasonal pulses of leaf litter inputs, a substantial quantity of dissolved organic matter (DOM), dissolved inorganic nitrogen (DIN) and soluble reactive phosphorous (SRP) is rapidly released into the streams due to the leaching process (Mcdowell and Fisher 1976; Wymore et al. 2015). Dissolved inorganic compounds from leachates are readily available for plant and microbial uptake in the stream without requiring mineralization and the metabolic costs of enzyme production (Sinsabaugh et al. 2002). Therefore, leaching from leaf litter inputs can substantially provide a suit of DOC, DIN and SRP to stream communities contributing to in-stream cycling of these elements. Despite the potential effect of leachates on the cycling of C, N and P in the recipient streams, the influence of leachates to streams has been a topic scarcely assessed in the literature and studies mainly focused on determining the loss of leaf litter mass associated to the leaching process (Brock T. 1984). The examination of how leachates influence on C, N and P in-stream pools and how these leachates react with microbial communities of the streams is still lacking (but see Wymore et al. 2015).

Microbial assemblages developed on leaf litter obtain C and N from leaf litter tissues and release part of these elements to the water column as leaf litter mineralization

proceeds. Webster and colleges (2009) suggested that microbial decomposers have a fixed C:N:P requirements with no stoichiometry plasticity. Therefore, the mineralization of leaf litter constituents can be direct, when the N and P supplied from leaf litter are greater than the needs of the microbes. Furthermore, litter mineralization can be indirect, which occurs when microbial assemblages metabolize the C from leaf litter and nutrients from leaf litter are released as inorganic nutrients to water column. Therefore, leaf litter decomposition implies the release of nutrients from leaf litter to the water column (Pastor et al. 2014). In addition, the linkage between leaf litter decomposition and nutrient stocks in water column is often associated to the microbial uptake of solutes from water column. In this regard, Kaushik and Hynes (1971) indicated that mineralization of leaf litter inputs is sustained by the uptake (or immobilization) of dissolved nutrients such as nitrogen. This process can be explained because microbial assemblages colonizing leaf litter are usually not completely satisfied only by compounds from leaf litter. Since Kaushik and Hynes (1971), other studies examined the use of dissolved nutrients by microbial assemblages colonizing leaf litter inputs. Most of these studies focused on determine N uptake from water column when adding N isotopically labelled ammonium (NH_4) or nitrate (NO_3) (i.e., $^{15}NH_4$ and $^{15}NO_3$), which barely modifies ambient nutrient concentrations (Dodds et al. 2000; Mulholland et al. 2000; Tank et al. 2000; Sobota et al. 2012; Ribot et al. 2017). These studies suggest that microbial decomposers take up inorganic N from the water column (Mulholland et al. 2000; Sobota et al. 2012; Ribot et al. 2017). However, these studies typically consider leaf litter as a bulk, despite the fact that leaf litter inputs are usually constituted by a set of leaf litter species with different quality, which is a factor that controls the microbial nutrient demands from water column (Webster et al. 2009). In parallel, some studies used stable isotopes of ^{13}C suggesting the assimilation of DOC

during leaf litter decomposition (Hall and Meyer 1998; Abril et al. unpublished data). Thus, leaf litter decomposition may be also related to the dissolved C dynamics in streams through the uptake of DOC by microbial decomposers.

The effect of dissolved nutrients on leaf litter decomposition has been also examined by modifying the background nutrient concentrations (Rosemond et al. 2015) or by considering streams covering a gradient of nutrient concentrations (Woodward et al. 2012). In a recent paper, Rosemond et al. (2015) used whole-stream nitrogen N and P additions to stream to test how nutrient enrichment can modulate leaf litter decomposition. They found that average decomposition of leaf litter was enhanced by ~50% as compared to reference conditions as a result of nutrient enrichment. Woodward et al. (2012) suggested that leaf litter decomposition can be influenced by dissolved nutrients across streams covering a gradient of nutrient concentrations. More specifically, they found that Gaussian-shape models best explained the relationship between litter decomposition and nutrient gradient. However, Woodward and colleges found this pattern only for total decomposition by analyzing both together, microbial and macroinvertebrate decomposition. Dramatically slowed breakdown at both extremes of the nutrient gradient indicated strong nutrient limitation in unaffected systems, potential for strong stimulation in moderately altered systems, and inhibition in highly polluted streams. Furthermore, Woodward et al. (2012) found that the effect of dissolved nutrient concentrations on leaf litter breakdown may be higher for high-quality litter such as alder than for low-quality litter such as oak. Yet, the interplay between dissolved nutrient concentrations and leaf litter quality and the main mechanisms explaining this interaction are still scarce for microbially-driven decomposition.

CHAPTER 2: OBJECTIVES OF THE PRESENT THESIS

The present thesis aims to provide knowledge about the main drivers that influence the dynamics of leaf litter inputs in stream ecosystems. On the one hand, we assume that the importance of leaf litter inputs to streams can be subjected to the retention of this subsidy within the recipient streams. Thus, as a specific objective, we assessed how the heterogeneity of water velocity within a stream reach can influence the retention, spatial distribution, fragmentation and decomposition of leaf litter inputs. In addition, this thesis aims to understand how the composition of the riparian forest, which determines the quality of leaf litter inputs, can ultimately influence the in-stream dissolved C and N dynamics. To approach this aim, we specifically examine how the leaf litter quality of different riparian tree species can influence: (I) the chemical-composition and microbial bioavailability of leaf litter leachates to streams (II) the uptake of DIN and DOC from water column by microbial decomposers during decomposition process and how it is related to the activity of microbial decomposers and, (III) how microbially-driven leaf litter decomposition vary among streams which covered a wide gradient of inorganic nutrient concentrations. The thesis is divided in the following 4 chapters which correspond to specific questions mentioned above. The chapters are organized following the order of leaf litter dynamics exposed in the introduction section (Figure 1.1.).

Chapter 5. *Spatial heterogeneity of water velocity drives the transport, spatial distribution, and processing of leaf litter in streams.*

This chapter examines how water velocity influences in-stream leaf litter dynamics at reach scale, including leaf litter retention, spatial distribution of leaf litter within the reach, and leaf litter decomposition and physical fragmentation.

Chapter 6. *Chemical and optical properties of different litter leachates influence in-stream nutrient pool and microbial activity.*

This chapter focuses on characterizing the leaf litter leachates from different riparian tree species. The main objectives are to explore how chemical and optical properties of the leachates vary among different litter sources, and how such potential variation can influence the activity of microbial assemblages in streams.

Chapter 7. *When leaf litter species matter, microbial uptake of ammonium and acetate from stream water during decomposition*

The present study aims to understand how the uptake of DIN (i.e., $N\text{-}NH_4$) and DOC (i.e., acetate) from water column vary among riparian leaf litter species which differ in the initial quality (i.e., C:N ratio) and among different stages of leaf litter decomposition. Moreover, we explore whether differences in $N\text{-}NH_4$ and acetate uptake among leaf litter species are related to the production of microbial activity of decomposers.

Chapter 8. *Responses of microbially-driven leaf litter decomposition to stream nutrients depend on litter quality.*

The aim of this chapter is to understand how microbially-driven decomposition of leaf litter from two riparian tree species differing in elemental composition (i.e., C:N ratio) varies among streams which cover a gradient of nutrient concentrations. More specifically, we evaluate: (i) leaf litter decomposition rates, (ii) leaf litter C and N content throughout the decomposition period, and (iii) microbial extracellular enzyme activities. These parameters are examined for alder (i.e., high-quality litter, low C:N) and sycamore (i.e., low-quality litter, high C:N) across streams.

CHAPTER 3: STUDY SITE

3.1. La Tordera Catchment

This thesis was conducted in the catchment of the river La Tordera (Catalonia, NE Spain; Figure 3.1.), with an area of 868.5 km^2 and dominated by siliceous geology. Climate in this region is typically Mediterranean, with warm, dry summers and mild, humid winters. At the highest elevations in the northern side of the catchment, local climate is very humid (>900 mm of annual precipitation) in the context of the area, whereas in the southern side local climate is relatively dry (<500 mm of average annual precipitation). Within this catchment, we selected different study sites in order to conduct the experiments mentioned in the previous section, but most of the research was conducted in a sub-basin of La Tordera, which is Font del Regàs. More specifically, chapter 5 was partially conducted in a reach of Font del Regàs. The litter material used in the laboratory experiment (chapter 6) was collected in the same reach. Chapter 7 was completely conducted in a canal adjacent to a stream reach within this sub-basin. Additionally, one of the streams used in the chapter 8 was placed in Font del Regàs and the other 4 streams were placed in other sub-catchments within La Tordera catchment (Figure 3.2.).

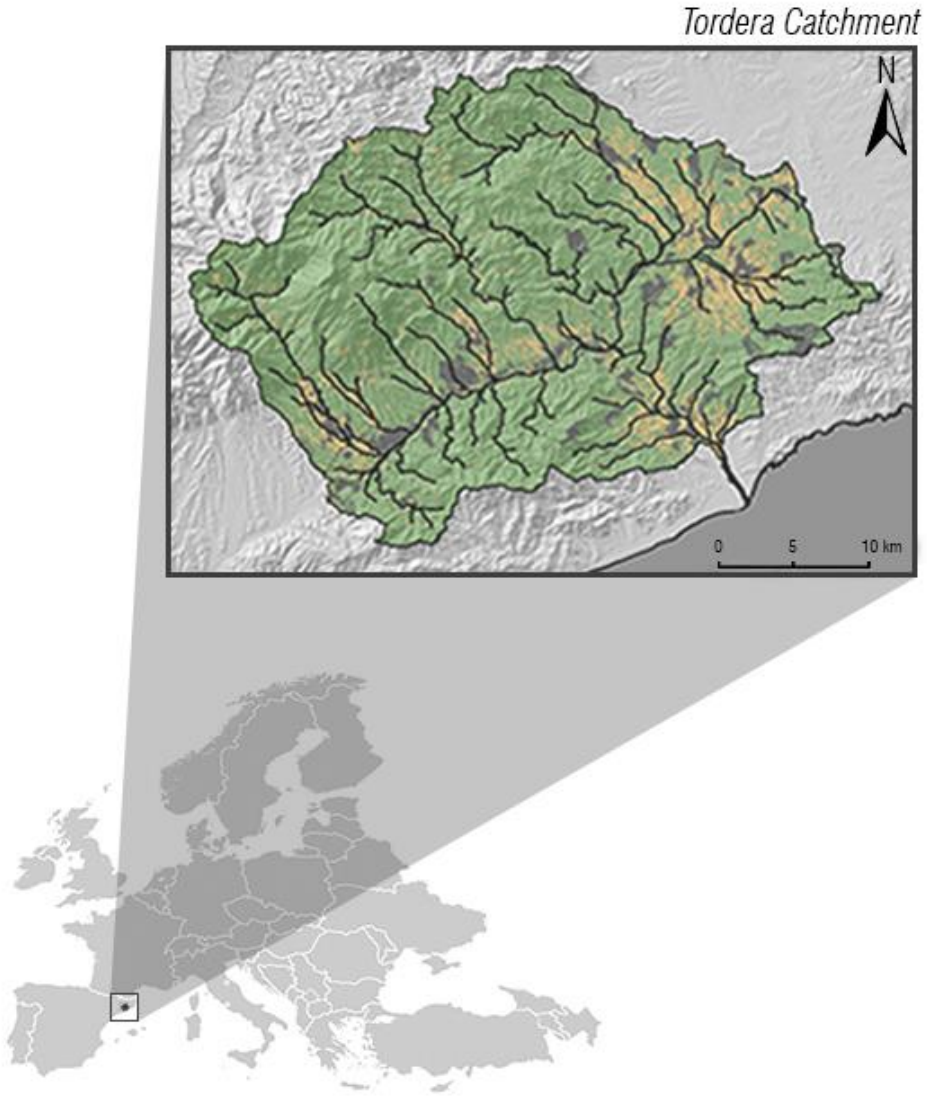

Figure 3.1. Location of La Tordera catchment (Catalonia, NE Spain).

3.2. Font del Regàs sub-catchment

Font del Regàs lies within the boundaries of the Montseny Natural Park (N of Barcelona; 41º50'N, 2º30'E, altitudinal range 300-1200 m a.s.l.), in the wettest part of La Tordera (Figure 3.2). Font del Regàs (12.5 Km^2) is highly forested, mostly dominated by sclerophyllous forest of evergreen oak (*Quercus ilex*), except in its hillslope northern part, which is dominated by a deciduous forest of beech (*Fagus sylvatica*). Riparian zones in Font del Regàs are constituted by a well-developed riparian forest, consisting mainly of alder (*Alnus glutinosa*), ash (*Fraxinus excelsior*), black poplar (*Populus nigra*) and black locust **(***Robinia pseudoacacia*). The study streams within this sub-catchment showed well-preserved channel morphology, with a riffle-run structure and low slopes (<5 %) along the reaches. The streambed is mainly composed by rock, cobbles, and gravels. The stream channel is, on average, 3-4 m wide. Study reaches are influenced by low human pressure and thus, are characterized by relatively low nutrient concentrations (von Schiller et al. 2008). However, two streams considered in the chapter 8 (Gualba and Coloma; Figure 3.2.) receive the inputs from wastewater treatment plants, and thus, these streams have higher nutrient concentrations and pollution.

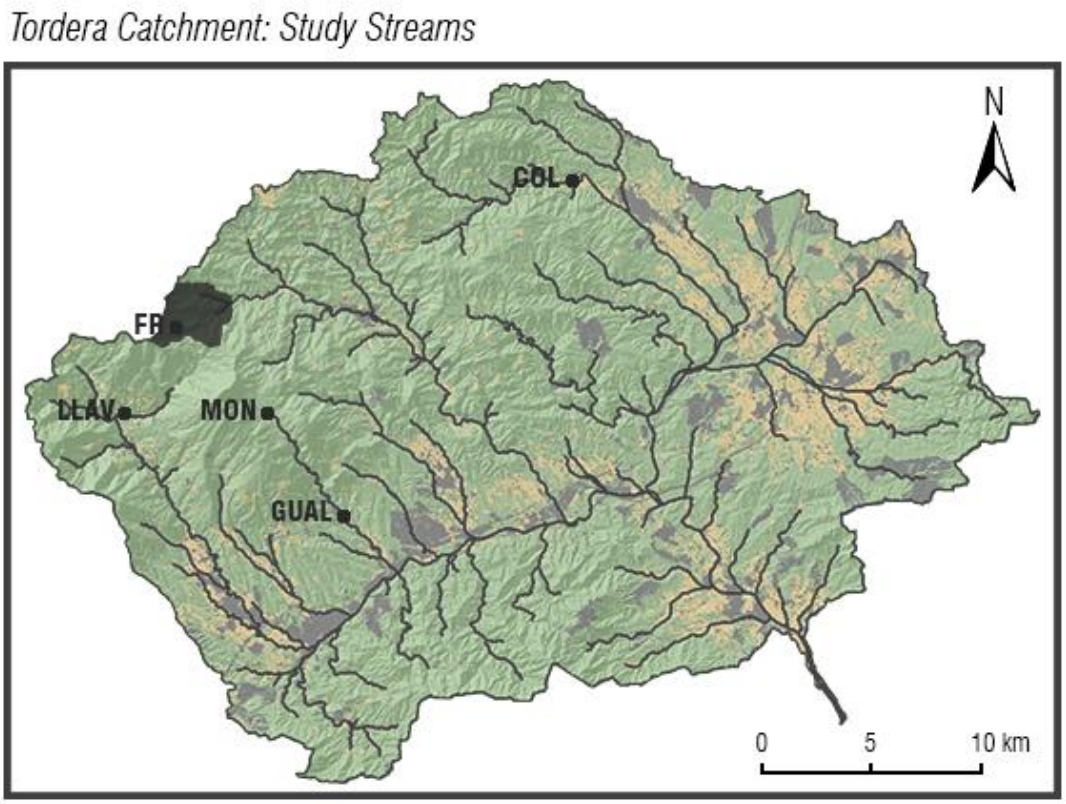

Figure 3.2. Location of the 5 streams study during the present thesis (chapter 8). We marked in bold Font del Regàs sub-catchment.

CHAPTER 4: EXPERIMENTAL TECHNIQUES

4.1 Experimental tracers used in the present thesis

The present thesis involved a set of field and laboratory methods to approach the objectives mentioned above. On the one hand, we used the leaf bag approach to calculate leaf litter decomposition rates (*k*) when needed. This technique is one of the most commonly used in leaf litter decomposition experiments in aquatic and terrestrial ecosystems (Webster and Benfield 1896). On the other hand, the present thesis entails the use of 4 different tracers in order to address the objectives mentioned above (Table 4.1.). More specifically we used: (a) tracer-leaves of *Ginkgo biloba* which was used to determine the retention and spatial distribution of leaves within a stream-reach. (b) Determination of dissolved organic matter quality by optical properties. (c) Microbial exozymatic activities, with are used to determine the degradation of specific organic compounds during leaf litter decomposition. (d) Resazurin (Raz)-resorufin (Rru) system, which allowed the estimation of microbial activity associated with each leaf litter leachate examined. (e) Stable isotopes of N (^{15}N-NH_4) and C (^{13}C-acetate) which allow determining the fluxes of these solutes from water column to the microbial decomposers (Table 4.1.).

Table 4.1. Different tracers used in the present thesis. We show a brief explanation of each tracer, the study area and scale at which they are used and the chapters where we used each tracer.

Experimental tracer	Uses	Study area	Chapters used
Determination of retention and spatial distribution of leaf litter inputs at reach scale	We quantified leaf litter retention and spatial distribution of leaves along a stream reach using leaves of *Ginkgo biloba* as tracer-leaves and adding them in a pulse into the reach. *Ginkgo biloba* leaves were used as a tracer of leaf transport and retention, because they can clearly be distinguished from the autochthonous leaves present in the stream channel	Field. Reach and within the reach scales	5
Determination of dissolved organic matter quality by optical properties	In the present thesis we used the specific ultraviolet absorbance at 254 nm (i.e., $SUVA_{254}$), which is being widely used as a proxy of the degree of humification, aromaticity, and molecular weight of DOM. Other recently indexes associated with DOM aromaticity that we used are the ratios E_2/E_3 and E_4/E_6	Laboratory assay	6
Quantification of the activity of microbial decomposers developed on leaf litter: exoenzyme activities	The expression of exoenzymes represents a useful tool to determine the microbial activity associated to the degradation of specific compounds. We measured two microbial enzyme activities mostly used in microbial-mediated leaf litter decomposition studies. The cellobiohydrolase activity as an indicator of leaf litter microbial degradation activity and especially for a recalcitrant compound such as cellulose and the phosphatase activity to assess how changes in the inorganic nutrient availability (i.e., SRP) may affect the potential microbial use of organic phosphorus compounds	Laboratory assay	5,7 and 8
Quantification of the activity of microbial decomposers developed on leaf litter: Raz-Rru system	The activity of microbial decomposers has been also analyzed by the Raz-Rru system. This system is a weakly fluorescent redox-sensitive dye that undergoes an irreversible reduction from Raz to strongly fluorescent Rru under mildly reducing conditions, most commonly in the presence of living microorganisms	Laboratory assay	6
Quantification of the leaf litter microbial uptake of dissolved inorganic nitrogen and dissolved organic carbon from water column	To measure demands of dissolved inorganic nitrogen (DIN) and dissolved organic carbon (DOC) from the water column by microbial assemblages on leaf litter we used stable isotopes of $^{15}N\text{-}NH_4$ and ^{13}C-acetate as tracers	Field. Reach and habitat scales	7

Figure 4.1. Addition of Ginkgo biloba leaves into a ~80m long reach (chapter 5). We recover the fraction of added leaves which reaches the net-trap placed at the end of the study reach (i.e., exported leaves).

4.2 Characterization of leaf litter inputs in Font del Regàs

We quantified the inputs of leaf litter to streams because senescent leaves were the main material used in the present thesis and thus, the dynamics of these inputs are important to understand the relevance of our conclusions. The quantification of leaf litter inputs was conducted during the period comprised from 2011 to 2014 (~80 sampling dates), which covered a remarkable range of hydric conditions. Leaf litter inputs were collected in a 100-m reach of Font del Regàs sub-basin with aerial traps (1 m^2, n = 5) placed over the stream-channel. The leaf litter samples were sorted into the dominant tree species (i.e., alder, ash, black locust and black poplar). After all leaf litter samples were classified, they were oven-dried (60 ºC during ~48 hours) and weighed (Sartorius, AX) to obtain the dry mass for each leaf litter type. These values were plotted against the Julian days to characterize the temporal patterns of each leaf litter species for each study year. Dry mass of each leaf litter type on each sampling date was divided by the number of days from the last collection and by the total area of the 5 aerial traps (5 m^2) to obtain daily rates of leaf litter inputs per stream reach area (mg DM m^{-2} d^{-1}) for each leaf litter type. We characterize the annual regime of leaf litter inputs for each dominant leaf litter type by fitting the daily rates of leaf litter inputs (I) to a Gaussian model:

$$I = A * e^{(-0.5) \cdot \left(\frac{x - x0}{b}\right)^2}$$

Where x is day of the year expressed in Julian days (where 1 is the 25th of January and 365 is the 24th of January of the following year); *A* is the maximum daily rate of inputs of leaf litter (in mg DM m^{-2} d^{-1}), which corresponds to the peak of inputs in the Gaussian model; *x0* is the day of the year when rates are maximum (day), and *b* is the amplitude of the curve when I is at half of the maximum value (in days).

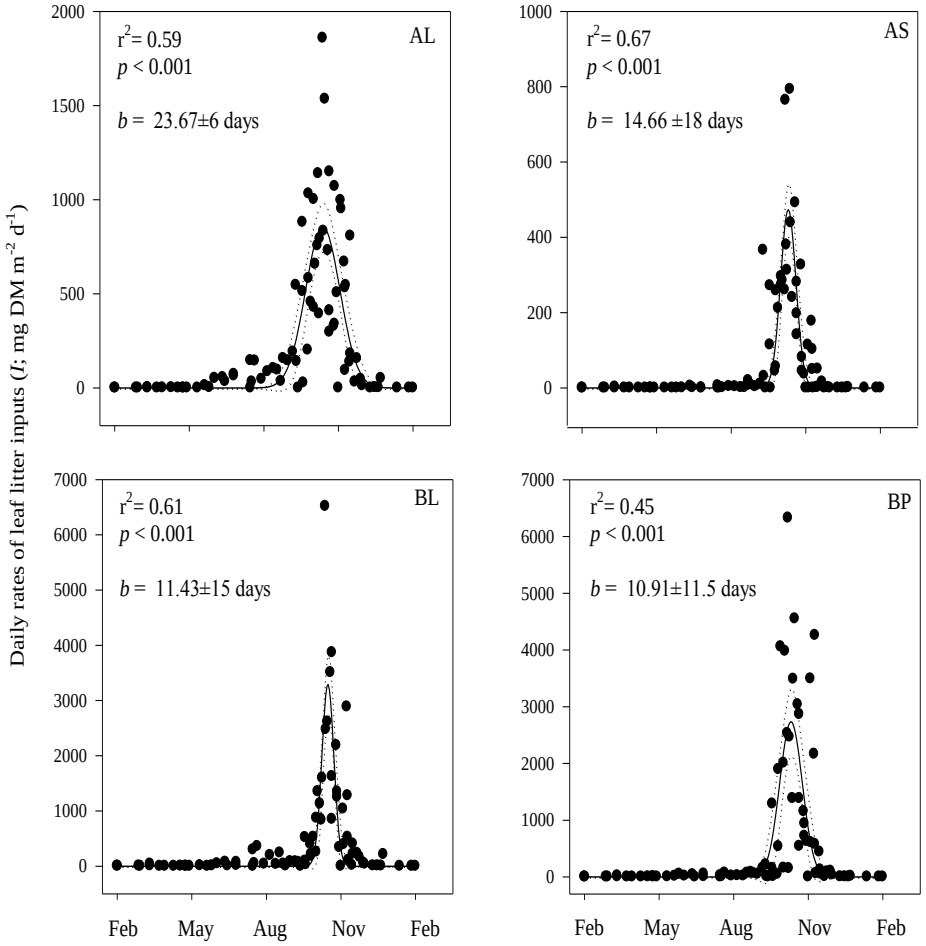

Figure 4.2. Temporal patterns of leaf litter inputs to streams for alder (AL), ash (AS), black locust (BL) and black poplar (BP). Data obtained from 2011 to 2014 was pooled together for every leaf litter species. Where *b* is the amplitude of the curve when the daily rates of leaf litter inputs is at half of the maximum value (in days).

Our results revealed that the day of the peak of leaf litter inputs (i.e., *x0*) ranged from 14th October to 26th November among species and years (data not shown). However, observed variability of *x0* was not significantly influenced neither by tree species nor years (ANOVA; $p > 0.05$). Thus, the composition of riparian forest and the particular weather conditions may scarcely influence when the peak of leaf litter inputs occurs. By the contrary, results indicated that composition of tree species in riparian zones can influence the length of period during which leaf litter inputs occurs. This fact was

supported by the observed variability of *b* among species (Figure 4.2.). In this regard, higher values of *b* were observed by alder species, indicating that alder had a wider period of leaf litter inputs than other riparian tree species studied, although significant differences were not achieve at 0.05 level (ANOVA, $p = 0.07$; Figure 4.2.). The variability of *b* among years was not significant (data not shown; ANOVA, $p = 0.14$). Therefore, the most relevant fact influencing the temporal pattern of leaf litter inputs to streams seems to be the presence of alder in the riparian zone, although significant differences were not achieved. Therefore, alder species not only provides high-quality substrate for in-stream microbial assemblages, but also could provide leaf litter into the stream during a wider time frame. This particular behavior of alder can have important implications for dynamics of organic matter of the recipient streams. In fact, in streams of the Mediterranean regions the timing of leaf litter inputs can coincide with floods events, which export most of the inputs to downstream ecosystems. Therefore, the presence of alder could mitigate the loss of leaf litter on recipient reaches, because part of the alder inputs can be provided long before or after the flood events.

CHAPTER 5: SPATIAL HETEROGENEITY OF WATER VELOCITY DRIVES THE TRANSPORT, SPATIAL DISTRIBUTION, AND PROCESSING OF LEAF LITTER IN STREAMS

5.1. Abstract

We conducted a holistic analysis of how water velocity influences in-stream leaf litter dynamics, including retention of leaf litter inputs, spatial distribution of retained leaf litter and decomposition within a reach. To tackle this objective, we combined a series of leaf tracer (i.e., *Ginkgo biloba*) additions with measurements of leaf decomposition (i.e., *Alnus glutinosa*) in different locations within a reach. In addition we characterized the microbial activity associated with decomposing leaves, using exoenzymatic techniques (i.e., Cellobiohydrolase activity), as a potential mechanism explaining potential spatial variation in decomposition rates. Our results demonstrate that the spatial variability of water velocity within a reach can influence the capacity of the reach to retain the inputs of leaf litter as well as the spatial distribution of these inputs in the reach. Our results also revealed that leaf litter decomposition can remarkably vary within a stream reach, and that this variability can be driven by the spatial heterogeneity of water velocity in the reach. In this regard we propose a novel method to standardize decomposition rates by the water velocity influences among different locations within the reach. The present study suggests that water velocity is a factor controlling different aspects of leaf litter dynamics in streams because not only influences the fraction of leaf litter retained but also how these inputs are spatially distributed and further processed within the streams. Therefore, this factor should be considered in future studies to increase our understanding of how leaf litter inputs can effectively fuel the metabolism of stream ecosystems.

5.2. The influence of water velocity on in-stream leaf litter dynamics

Riparian forests provide substantial allochthonous subsidies of organic matter to headwater streams (Cummins 1974, Webster and Benfield 1986; Lamberti and Gregory, 1996). One of the most important components of this subsidy is leaf litter, which provides substrata, organic carbon and nutrients (i.e., nitrogen and phosphorus) to in-stream microbial communities. Thus, leaf litter inputs contribute to stream metabolism (Fisher and Likens 1973; Vannote et al. 1980), nutrient cycling (Mulholland et al. 1985; Hall and Meyer 1998; Valett et al. 2008), and influence food web composition (Webster et al. 2000).

Early recognition of the importance of leaves to stream ecosystems has led to a rich literature addressing the factors that control the rate of leaf breakdown and decomposition (*k*). Intrinsic factors of the leaves, such as leaf fiber content, chemical inhibitors of microbial decomposers, and the absolute and relative abundance of nutrients in leaf tissue, are shown to influence *k* (Webster and Benfield, 1986). In addition, several extrinsic factors have also been identified as important drivers of leaf litter decomposition, including temperature (Ferreira and Chauvet 2011), water column nutrient availability (Woodward et al. 2012), oxygen concentration (Webster and Benfield, 1986), and water velocity (Witkamp and Frank 1969; Hodkinson 1975). However, most of these studies estimate *k* based on measurements done at a specific stream location (i.e., plot-scale perspective), ignoring the likelihood that leaves can be distributed at several locations within the stream channel (i.e., reach-scale perspective). Therefore, these studies implicitly assume that plot-scale measurements of leaf litter decomposition can be up-scaled at ecosystem level, without considering that leaf litter decomposition can spatially vary due to the spatial heterogeneity within the ecosystem.

As an example, for a given stream reach the interaction between flow and streambed morphology generates a wide mosaic of water velocities. This factor has been shown to influence leaf litter decomposition. In this sense, Ferreira et al. (2006) already recognized that water velocity can influence *k* through physical abrasion. Moreover, water velocity has been often related with increases in the activity, reproduction and colonization of fungi inhabiting on leaf litter because water velocity enhances water turbulence; and thus, the oxygen available for microbial decomposers (Canhoto et al 2013). However, Ferreira and Graça (2006) reported more diverse fungal communities under low flow conditions, suggesting a negative effect of water velocity on the community composition of microbial decomposers. Therefore, since water velocity can be an important driver of leaf litter decomposition in streams, examining how the heterogeneity of water velocity in the stream channel could influence the spatial variability of leaf litter decomposition at reach scale can help understanding and up-scaling this process at ecosystem level.

Water velocity is also an important factor controlling the capacity of streams to retain leaf litter inputs (i.e., retentiveness). In this regard, several studies observed that retentiveness is inversely related to the average stream velocity and discharge (Snaddon et al.1992, Raikow et al. 1995, Dewson et al. 2007). This fact was explained because high stream velocities create bed shear stresses and water column turbulence that re-suspend benthic organic matter and reduce particle deposition. Therefore, high stream velocity conditions result in longer transport distances of leaf litter inputs (Fisher and Likens 1973, Larrañaga et al. 2003, Cordova et al. 2008). At low to moderate velocities, leaf litter strongly interacts with streambed substrate. Thus, leaf litter tends to buildup on the streambed where shear stresses are sub-critical (e.g. pools) or where leaves

become trapped by streambed obstacles such as wood, cobbles or boulders (Larrañaga et al. 2003, Cordova et al. 2008). Furthermore, leaf litter distribution within a reach is not a static phenomena because leaves often experience successive deposition-resuspension cycles whenever stream velocity conditions shift, which generates a shifting mosaic (Fisher and Likens, 1973). Considering these observations, we argue that the spatial heterogeneity of water velocity within a reach would influence the retention and the spatial distribution of retained leaf litter, because leaves can either be retained or transported depending on the shear stress conditions of the streambed locations where they interact (Nakajima et al. 2006).

Since water velocity can influence the balance between transport and retention, the spatial distribution of retained leaf litter and their decomposition rates, research that simultaneously considers the effect of this factor on these processes is required to better understand leaf litter dynamics at reach scale. To this aim, we conducted a holistic analysis of how water velocity influences in-stream leaf litter dynamics, including retention of leaf litter inputs, spatial distribution of retained leaf litter and decomposition within a reach. To tackle this objective, we combined a series of leaf tracer additions with measurements of leaf decomposition in different locations within a reach. In addition we characterized the microbial activity associated with decomposing leaves, using exoenzymatic techniques, as a potential mechanism explaining potential spatial variation in *k*. We hypothesized that spatial heterogeneity of water velocity within a reach will explain the distribution of retained leaf litter because velocity controls the local dynamics of leaf litter re-suspension and deposition from sites of high velocity to those of low velocity. In addition, we hypothesized leaf litter *k* will vary within the reach in relation to water velocity because this factor influences both the

physical fragmentation of leaves and the composition and activity of microbial decomposers. We considered that environmental factors influencing the activity of microbial decomposers such as temperature and water chemistry would be similar among sampling locations within the reach; whereas other factors such as oxygen concentration could spatially vary with water velocity because it can be depleted under null velocities due to the lack of water turbulence. Therefore, we expect that the activity of aerobic microbial decomposers colonizing leaf litter would be equal among sampling locations, if all locations are well oxygenated. If not, aerobic microbial activity associated to low-velocity habitats will be lower because of the reduction of the oxygen concentration; which may lead to low leaf litter *k* in these zones.

5.3. Methods

Field methods

Leaf litter additions to estimate retention and spatial distribution in the study reach

The influence of water velocity on retention of leaf litter inputs and their spatial distribution within the recipient reach was evaluated in a 70 m long and 3.5 m wide reach of the Ebron headwater stream located near Tormón village (Teruel, E of Spain; 40º 20' N, -1.35W; 1051 m a.s.l.). We quantified leaf litter retention in the study reach by adding 200 leaves of *Ginkgo biloba* at the top of the reach and consecutively follow the transport of these leaves along the reach. A plastic net (1 cm of mesh size) was placed at the end of the reach to trap leaves being exported from the reach. *Ginkgo*

biloba leaves were used as a tracer of leaf litter inputs, to distinguish the added leaves from the autochthonous leaves that were already present in the stream channel and be able to empirically estimate leaf litter retention (Pozo et al. 2009). During this study, we performed 4 leaf litter additions, each one differing in the elapsed time between leaf addition and collection of leaf litter along the stream (i.e., at 1, 20, 70, and 490 hours after the leaves were added) to examine both retention and distribution of leaves within the stream reach. Differences in collection times among leaf litter additions aimed to estimate potential effects of spatial re-distribution of leaves within the reach over time. On each leaf litter addition, and for each leave of *Gingko* added to the stream reach we measured the distance travelled along the reach and the water velocity at the location where the leave was retained (5 measurements of water velocity at mid-depth per sampling site) using a velocity meter (Miniair20/Schiltknecht). In addition, to characterize the study reach in terms of spatial heterogeneity of hydro-morphological characteristics, we measured wetted channel width at 3 m intervals along the reach (23 transects) and velocity at every 20 cm across each transect (342 point measurements).

Effect of velocity on leaf litter decomposition rate within the reach

The influence of water velocity on rates of leaf litter decomposition at reach scale was evaluated in a 100 m long and 4 m wide reach in Font del Regàs, a 3rd order stream within La Tordera catchment (N of Barcelona, Spain; 41°50´ N, 2°30´ E; 300 m a.s.l.). For this study, we used leaf litter from alder (*Alnus glutinosa*), the most common riparian tree species at the study reach. Alder leaves were collected during the peak fall (i.e., mid-November 2013) using traps placed over the stream channel. To measure rates of leaf litter decomposition (*k*), 3 g of air-dried leaves were placed in 250-µm mesh-size

bags, which mostly excluded macroinvertebrates; and thus basically allowed measurement of decomposition rates associated with microbial activity. Leaf litter bags were deployed at 8 locations within the reach, which covered a range of water velocities from ~0 to 92 cm s^{-1}. Leaf bags were incubated in the stream from the 1st February to the 28th March 2014. During this period, leaf bags were collected on 5 sampling dates, i.e. 2, 7, 14, 40 and 57 days after deployment in the stream (4 replicates per sampling location and sampling date). An additional set of leaf bags (4 replicates per sampling location) was collected after 55 days of incubation to quantify the extracellular enzyme activity of cellobiohydrolase (CBH; EC 3.2.1.91) following the procedure by Romaní et al. (2006). During this decomposition time, it was expected that leaf litter packs had roughly loosed 40–60% of their initial mass. The CBH activity was measured as an indicator of the microbial activity specially associated with the leaf litter degradation of recalcitrant compounds such as cellulose. We expected that microbial assemblages were well developed after 55 days of incubation and that the CBH enzyme activity was representative of leaf litter decomposition (Romaní et al.2006). Once collected, leaf bags were kept cold (~4ºC) to be transported to laboratory.

During the incubation period, water temperature and stream water level were recorded every 20 minutes using 5 waterproof temperature data loggers evenly distributed along the reach (HOBO Pendant® UA-002-64) and a pressure data logger placed at the bottom of the reach (Solinst Levelogger Junior Edge). Every 5 days and on each date of leaf bag collection, water velocity, water depth, and dissolved oxygen concentration were measured at each leaf bag sampling location (5 measurements per location). Reach-scale measurements of stream discharge on each sampling date were done using a mass balance approach by adding 1 L of NaCl-enriched solution to the channel (Gordon et al. 2004). We used the relationship between discrete measurements of discharge and daily

values of stream water level to infer daily values of stream discharge for the entire study period. Then, we also used this relationship to infer daily values of water velocity at each leaf bag location during the entire study period. Finally, on each date of leaf bag collection, we collected water samples at 3 sites along the reach (top, middle and bottom of the reach) for analyses of the concentration of ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$), and soluble reactive phosphorus (SRP). Analysis of nutrient concentrations was determined following standard colorimetric methods (Apha 1995) on an Automatic Continuous Flow Futura-Alliance Analyzer at the Nutrient Analysis Service of the Centre d´Estudis Avançats de Blanes (CEAB), Barcelona, Spain.

Laboratory analyses and data calculation

Retention and spatial distribution of leaf litter within the reach.

Measured distances travelled by added leaves that were retained along the study reach were grouped into 35 categories (i.e., at 2 m intervals along the 70 m reach) to cover the total length of the reach. The leaf retention coefficient per unit distance along the reach (k_x, in m^{-1}) was estimated based on the amount of leaves retained along the streambed of the study reach by fitting empirical data to the exponential model:

$$L_x = L_{x0}\, e^{-kx\, x} \qquad (1)$$

Where L_x is the number of retained leaves found at each x (m) distance from addition point and Lx_0 is the estimated number of leaves retained at 0 m from addition point. The inverse of k_x (i.e., S_W in m) is the average distance travelled by leaves along the reach before being retained in the streambed. In all additions, some added leaves could not be visually found either within the reach or at the end of it in the net. This caused that the number of retained leaves found within the reach plus the number of leaves trapped in

the net (F_{leaves}) was less than the total number of leaves added (A_{leaves}). Therefore, we calculated a percentage of leaf recovery (PR), estimated following equation 2, as an indicator of the reliability of the data derived from the leaf additions.

$$PR = (F_{leaves} / A_{leaves}) 100 \quad (2)$$

In addition, we examined the relationship between the number of retained leaves and the particular velocity at each retention site to examine how water velocity influences leaf distribution within the reach. To approach this relationship, the range of water velocities measured at all retention sites (i.e., from ~0 to 90 cm s^{-1}) was grouped within velocity intervals of 5 cm s^{-1}, resulting in a total of 18 categories. We found that the best fit describing this specific relationship was the following exponential decay model

$$L_v = Lv_0 \, e^{-kv \, v} \quad (3)$$

where L_v is the number of leaves retained at each water velocity category v (cm s^{-1}), Lv_0 is the estimated number of leaves retained under the water velocity category of 0 cm s^{-1}, and k_v is the leaf retention coefficient per unit of water velocity along the reach (s cm^{-1}). k_v represents the fractional change in the number of leaves found at habitat scale in a given reach with increasing water velocity. The inverse of k_v (cm s^{-1}) is an indicator of the average water velocity at which leaves are retained in the reach. This exponential model was also used to estimate the predicted number of leaves retained at each velocity category within the reach. To do that, the total number of leaves retained within the reach was multiplied by the relative proportion of stream-locations of each water velocity category. Then, for each addition of leaves, we calculated observed/predicted ratios in every water velocity category in order to explore whether the number of retained leaves followed the spatial patterns predicted from the water velocity mosaic measured into the reach. If leaves re-distributed within the reach over time randomly

with respect to the water velocity mosaic then we would expect that, after longer times since the addition, the observed/predicted ratios would become closer to 1 at each water velocity category.

Additionally, using data from the 4 leaf additions, we examined the degree of heterogeneity in the spatial distribution of the retained leaves in the reach and how it was related with water velocity distribution. To do that, we calculated the Euclidean distances in the number of retained leaves among the different category distances (n = 35). A total of 595 paired combinations among all category distances were calculated. The matrix of the standard deviation (SD) of the Euclidean distances was used as a measure of heterogeneity in the distribution of retained leaves for each addition; with higher SD values representing a higher heterogeneity. For each addition, we also examined the degree of heterogeneity in the spatial distribution of the retained leaves across the velocity range by calculating the Euclidean distances in the number of retained leaves among water velocity categories (n = 18). A total of 153 paired combinations among all categories of velocities were calculated. In this case, the SD of the Euclidean distances matrix was used as a measure of the influence of water velocity on the spatial distribution of retained leaves, with lower SD indicating lower influence of velocity. Differences in SD for distance travelled and retention velocity among the 4 additions provided information on the dynamics of spatial re-distribution of leaves within the reach and how velocity affected them.

Leaf litter decomposition rates within the reach.

Collected leaf bags from each sampling location were first rinsed with stream water to remove inorganic sediments attached to the bag. Then, leaf litter samples were carefully

removed from the bags and rinsed with stream water to remove inorganic sediments attached to the leaf litter surfaces. Leaf litter samples were oven dried (60 ºC during 48 hours) and weighted (Sartorius, AX) to obtain the remaining dry mass, which was expressed as percentage from the initial dry mass.

To estimate rates of leaf litter decomposition (*k* in d^{-1}) at each location, which denotes the velocity at which leaf litter mass decreases over time, the remaining dry mass on each sampling date was plotted against time following the model described by Petersen and Cummins (1974)

$$W_t = W_0 * e^{-k\,t} \qquad (4)$$

where W_0 and W_t are leaf litter dry mass (g) at the beginning and at sampling dates, respectively, t (days) is the incubation time.

To explore the influence of water velocity on leaf litter *k*, we summed the daily water velocity values measured over the decomposition period at every sampling location where leaf bags were deployed. The percentage of remaining dry mass of alder leaf litter was then plotted against cumulative water velocity on each sampling date at each location using the exponential decay model from equation 4 in a similar manner as that used to correct for the temperature effect in degree day (dd^{-1}) (Minshall et al. 1983). Therefore, for each stream location, we obtained a leaf litter decomposition rate standardized by local water velocity, which was expressed by velocity day (i.e., velocity standardized-*k*, in velocity-$days^{-1}$).

Exoenzymatic activity of decomposing leaf litter

We measured the CBH activity of leaf litter incubated over 55 days at different water velocity locations in the study reach using methylumbefelliferone (MUF) fluorescent-linked substrates, following the method described in Romaní et al. (2006). This assay was conducted at saturation substrate conditions of 1 mM. Leaf litter discs (14 mm diameter) from each velocity location (n = 4 per location) and water controls were incubated with the MUF-linked substrates for 1 h in the dark in a shaker (50 rpm). Blanks and standards of MUF (0-100 µmol L^{-1}) were also incubated. At the end of the incubation, Glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 365/455 nm excitation/emission (Spectrofluorophotometer Shimadzu/ RF-5000). Results of extracellular enzyme activities of CBH were expressed as the amount of MUF substrate produced per incubation time (h) and dry mass of leaf litter (g).

Statistical analysis

Retention and spatial distribution of leaf litter within the reach

We used analysis of covariance (ANCOVA) to explore differences in the leaf retention coefficients per unit distance (k_x) among the 4 leaf litter additions. The number of leaves retained in the reach was log-transformed prior the analysis to meet normality and homogeneity of variance assumptions. The ANCOVA model includes number of leaves retained as a dependent variable, the distance from addition point as the covariate, and each addition (n = 4) as a fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine specific differences in k_x among additions. We also used ANCOVA models to explore differences in leaf retention coefficient per unit of water velocity (k_v) among the 4 leaf litter additions. The

number of leaves retained in the reach was log-transformed prior the analysis to meet normality and homogeneity of variance assumptions. The ANCOVA model includes number of leaves retained as a dependent variable, water velocity at the location of the leaf litter retained as the covariate, and each addition (n=4) as a fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine specific differences in k_v among additions.

Leaf litter decomposition rates within the reach

We used one-way analysis of variance (ANOVA) with repeated measures (RM) to determine differences in water velocity, depth, dissolved oxygen concentration and temperature among sampling locations during the study period. The ANOVA model includes these parameters as dependent variables and stream locations (n = 8) as fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine specific differences in these parameters among sampling locations. We used ANOVA models to explore differences on CBH microbial activity among sampling locations. The ANOVA model includes CBH as dependent variable and stream locations (n = 8) as fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine specific differences in CBH among sampling locations.

To explore differences in *k* (day^{-1}) among sampling locations, we used ANCOVA analysis with log-transformed values of leaf litter remaining mass as a dependent variable, the incubation time (expressed in days) as the covariate, and sampling location as a fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine differences in *k* among locations. We also used ANCOVA

model to explore differences in *k* (expressed per velocity days) among locations. In this case, we used the accumulated water velocity values over the study period as the covariate.

To examine the effects of water velocity on leaf litter *k* (expressed both per days and velocity days) and on the CBH activity, we used linear and exponential regression analysis.

Statistical analyses were done with PASW Statistics 18 (v18.0.0/SPSS Inc).

5.4. Results

Retention and spatial distribution of leaf litter within the reach

During the addition of leaves, stream discharge was relatively low and constant (20 ± 2 L s^{-1}). The proportion of streambed locations influenced by low water velocities (< 10 cm s^{-1}) accounted for 70% of the total number of locations where water velocity was measured within the reach (Table S5.1.; see annexes section). The proportion of locations influenced by water velocities ranging from 11 to 90 cm s^{-1} accounted for 30% of total number of locations (Table S5.1.; see annexes section). Moreover, we observed that the percentage of stream locations associated with each water velocity category declined exponentially with water velocity, based on our intensive survey of water velocity within the reach (Table S5.1.; see annexes section). The percentage of leaves recovered (PR) from the additions ranged from 86% to 99%, making more reliable the spatial parameters calculated from retained leaves. In this regard, all additions of leaves resulted in significant exponential declines of retained leaves with distance (k_x) (Figure

5.1.). Moreover, the average distance travelled by leaves ($S_W = 1/k_x$) increased significantly with increasing the elapsed time between leaf addition and collection (ANCOVA, $p < 0.001$; Figure 5.1.), indicating that retained leaves re-distribute themselves over time and travel longer distances.

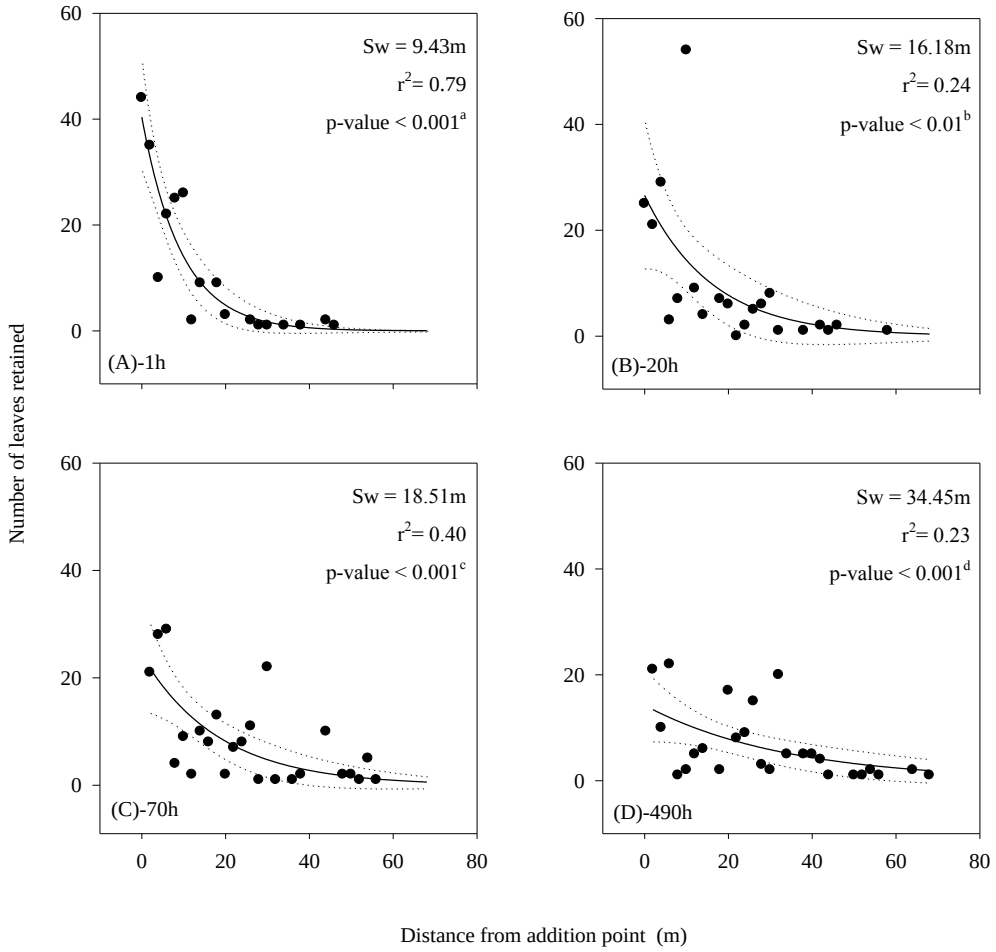

Figure 5.1. Relationship between the number of leaves retained within the stream channel and the distance from addition point for the 4 additions of leaves, which varied in the elapsed time between the addition and collection of leaves within the reach (from 1 to 490 h after leaf addition). Average travel distance ($S_w=1/k_x$) was calculated by fitting these relationships to negative exponential models. Letters next to the p-value of the regression indicate statistical differences in k_x based on ANCOVA analysis followed by post-Hoc Tukey´s t-test.

In agreement, the SD of retained leaves among distance categories was smaller as the elapsed time increased (Figure 5.2. A), indicating that leaves were more homogenously distributed within the reach over time.

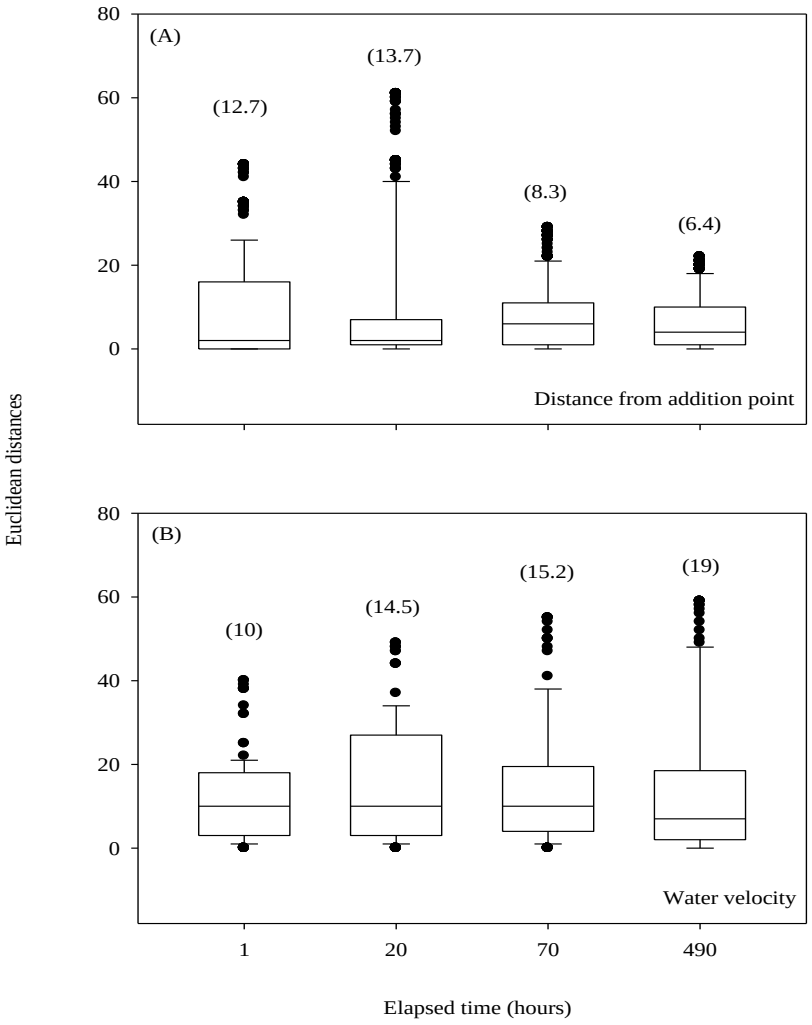

Figure 5.2. Results of standard deviation (SD, in parenthesis) of Euclidean distances representing the variability of retained leaves along the study reach (A) and across the velocity range (B). Note that low SD indicates more similar number of retained leaves among distance categories (A) or velocity categories (B), respectively. High SD values indicate greater heterogeneity in the amount of leaves retained along the reach (A) and across velocity range (B).

The spatial distribution of leaves within the reach was also significantly associated with overlying water velocity. In particular, all additions of leaves resulted in significant exponential declines of retained leaves with water velocity (k_v) (Figure 5.3., left panels). Moreover, the average retention velocity ($1/k_v$) decreased significantly with increasing the elapsed time (ANCOVA, $p < 0.001$; Figure 5.3., left panels), indicating that during the spatial re-distribution of leaves they were predominantly retained at locations with low water velocity. In agreement, results from the SD of retained leaves among velocity categories was higher as the elapsed time increased (Figure 5.2.B), indicating that leaves were more heterogeneously distributed across the velocity categories over time. In addition, if retained leaves were distributed randomly with respect to the water velocity mosaic observed within the reach, then the expected number of leaves retained in each velocity category would be proportional to the relative abundance of locations within each velocity category (i.e., observed/predicted ratios ~1; Figure 5.3., right panels). In this regard, the number of leaves retained in the slowest and highest velocity categories was generally similar or lower than expected by the velocity mosaic within the reach. In contrast, observed abundance of retained leaves was generally higher than expected at intermediate water categories (20 to 50 cm s^{-1}) (Figure 5.3., right panels). Even after more than three weeks of the elapsed time, leaf distribution in the study reach was still right-skewed toward mid-velocity zones relative to expectations based on the velocity distribution found within the reach. We used a power function relating k_v and the elapsed time in hours ($k_v = 0.026\ time^{0.181}$, $r^2 = 0.97$) to estimate that it would require approximately 15 years for the observed leaf distribution to match with the expected leaf distribution based on the relative abundance of locations within each water velocity category.

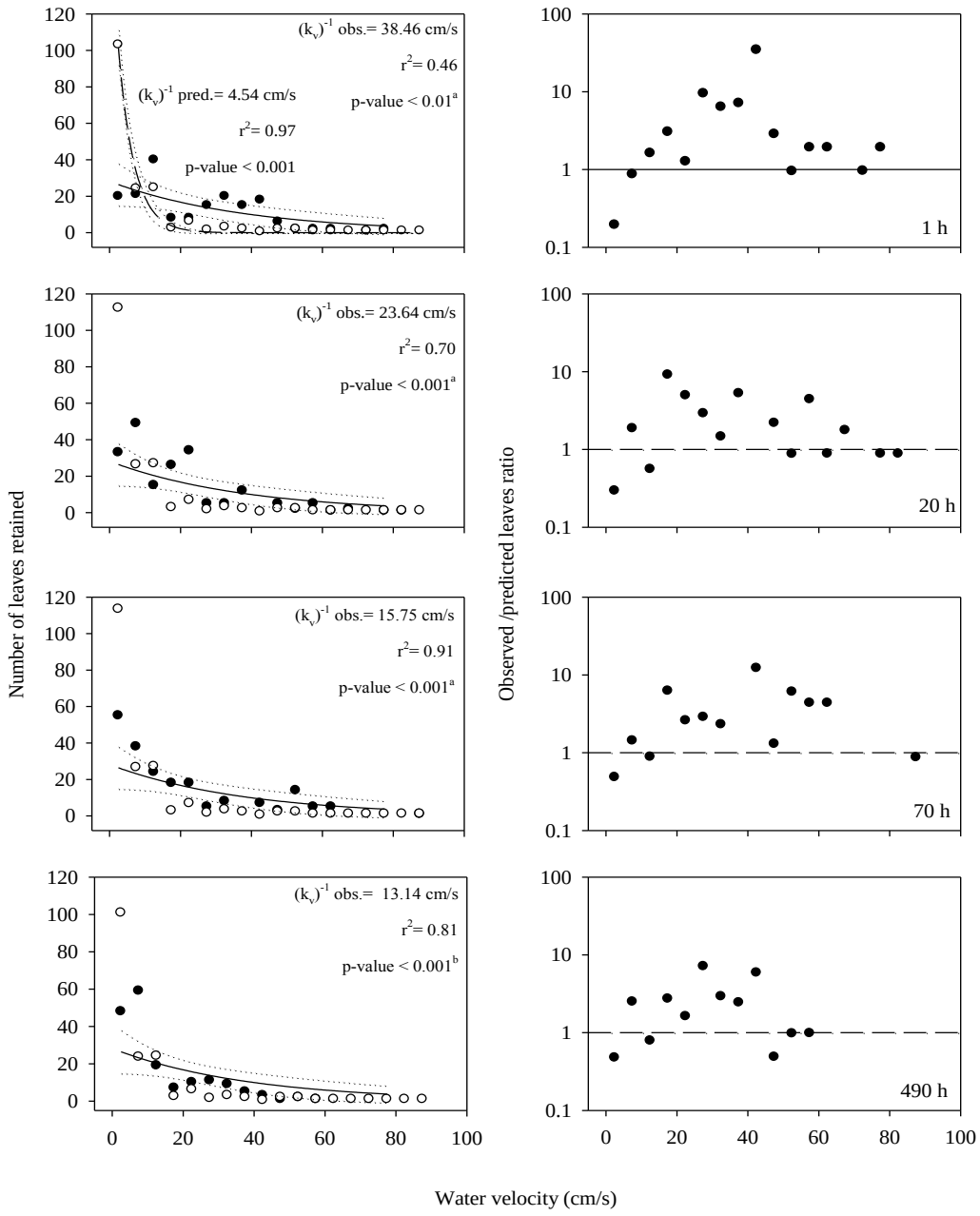

Figure 5.3. Distribution of leaves retained in the reach in relation to the different water velocity for the 4 additions which varied the time between leave addition and collection (from 1 to 490 h after leave addition). The coefficient k_v represents the leaf retention coefficient per unit of velocity. Dashed line shows the expected leave retention regression based on the proportion of water velocity locations within the reach $(k_v)^{-1}$ predicted. Filled and open points represent the observed and expected number of retained leaves found in every water velocity category, respectively (see text for further explanation). Letters next to the p-value of the regression indicate statistical differences on k_v among the 4 additions respectively, based on ANCOVA analysis followed by post-Hoc Tukey´s t-test (Left panel). Ratios between the observed number of leaves retained in each water velocity category and those expected by the relative proportion of water velocity measured within the reach were calculated (Right panels). Note that horizontal line (1) indicates the number of leaves observed was similar than those expected from within reach water velocity mosaic (Right panels).

Decomposition rates and microbial activity of leaf litter

During the period of leaf litter incubation, water discharge decreased from 60 to 50 L s^{-1}. However, water velocity and depth measured in each specific sampling location remained quite stable throughout the incubation time (ANOVA-RM, $p > 0.05$, data not shown). Nutrient concentrations were relatively stable during leaf litter incubation (ANOVA-RM, $p > 0.05$ (average ± SEM; n = 18): $N\text{-}NO_3^-$ + $N\text{-}NO_2^-$ (µgN L^{-1}) = 226 ± 22; $N\text{-}NH_4^+$ (µgN L^{-1}) = 12 ± 5 and SRP (µgP L^{-1}) = 11 ± 2). Furthermore, among sampling locations, water temperature and oxygen concentration were relatively similar (ANOVA-RM, $p > 0.05$) (Table 5.1.). We found significant differences in water velocity and water depth among locations (ANOVA-RM, $p < 0.001$), ranging from 0 to 92 cm s^{-1} and from 12.2 to 20.2 cm, respectively (Table 5.1.).

Table 5.1. Characteristics of the different sampling locations within the study reach where leaf bags were incubated during 57 days. Values are means of measurements done during the study period (n=12 sampling dates). Values in brackets represent the standard error of the mean associated with the spatial variation within the reach. Cumulative water velocity is the sum of daily water velocity during the entire study period on each location. Different letters indicate significant differences among locations for a given variable based on the results from one-way ANOVA analysis with repeated measures (i.e., different dates) followed by post-Hoc Tukey´s t-test.

Sampling locations	Temperature (ºC)	Dissolved O_2 concentration (mg L^{-1})	Depth (cm)	Water velocity (cm s^{-1})	Cumulative water velocity (cm s^{-1})
1	8.6^{a} (0.37)	9.5^{a} (0.74)	12.2^{a} (0.52)	0^{a} (0)	0
2	8.0^{a} (0.28)	10.6^{a} (0.99)	22.0^{d} (0.19)	7^{b} (3)	420
3	8.5^{a} (0.51)	10.7^{a} (0.14)	12.4^{a} (0.64)	15^{c} (3)	900
4	8.2^{a} (0.34)	10.7^{a} (0.14)	15.4^{b} (0.32)	29^{d} (2)	1660
5	8.6^{a} (0.53)	10.6^{a} (0.12)	14.8^{b} (0.31)	50^{e} (2)	2900
6	8.6^{a} (0.56)	10.7a (0.20)	12.2^{b} (0.39)	53^{e} (4)	3050
7	8.4^{a} (0.35)	10.8^{a} (0.17)	20.2^{b} (0.43)	78^{f} (3)	4510
8	8.1^{a} (0.37)	10.8^{a} (0.14)	19.4^{c} (0.52)	92^{f} (5)	5470

Mass loss of leaf litter during the initial days of decomposition (i.e., 2, 7 and 14 days) was similar across the range of water velocities examined (Figure 5.4.). In contrast, after day 40, mass loss of leaf litter differed among sampling locations, being higher at locations with higher water velocity. Mass loss differences among locations were largest on day 57, when mass loss was ~62% at locations with low velocity (close to 0 cm s^{-1}) and 75% at locations with high velocity (92 cm s^{-1}; Figure 5.4.).

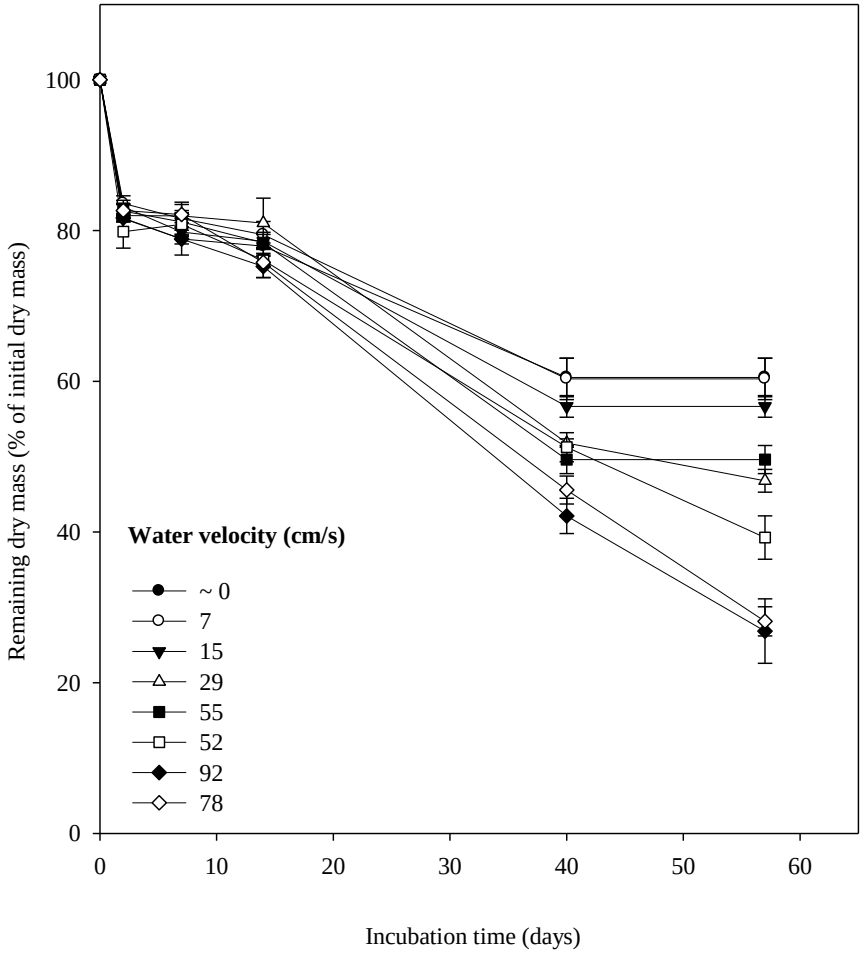

Figure 5.4. Temporal variation in the remaining dry mass (expressed as % of initial dry mass) of alder leaf litter during 57 days of incubation. Each line shows the temporal variation of leaves incubated under different water velocity conditions (n = 8; 0 - 92 cm s^{-1}).

Remaining mass of leaf litter over the incubation period was significantly fitted to the exponential decay model at all sampling locations ($0.77 < r^2 < 0.96$, $p < 0.0001$, Table S5.2. see annexes section). Values of leaf litter *k* significantly differed among sampling locations (ANCOVA, $p < 0.001$) and were positively related to water velocity at each location ($r^2 = 0.96$; $p < 0.001$; Figure 5.5. A).

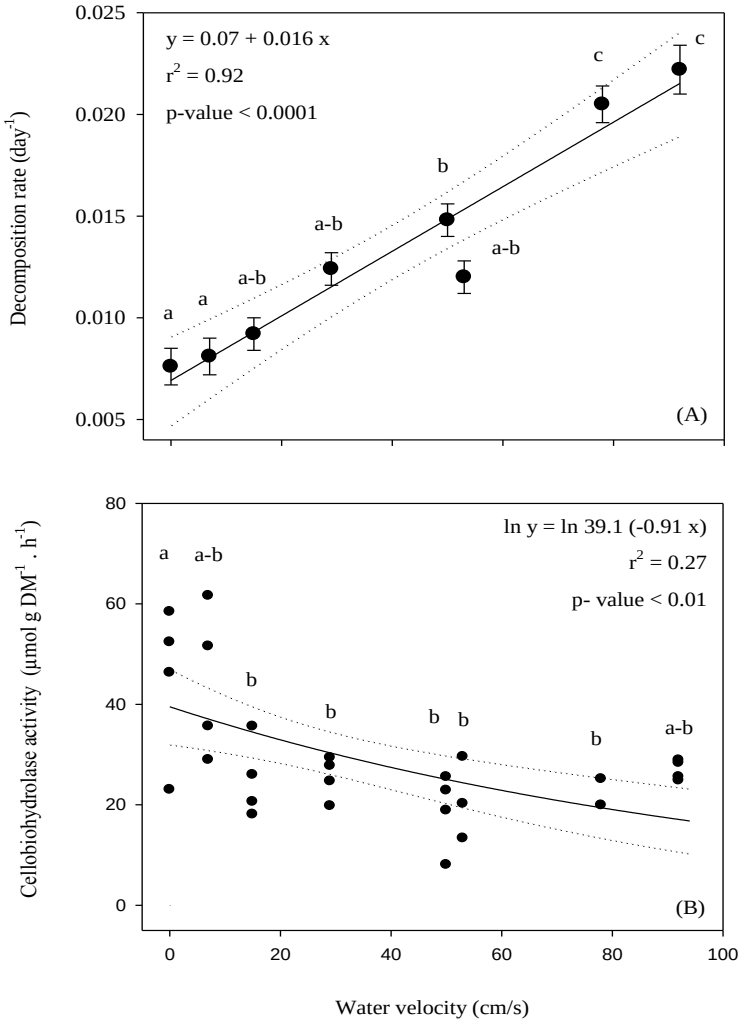

Figure 5.5. Relationships between (A) leaf litter decomposition rates and (B) microbial enzyme activity of cellobiohydrolase and water velocity. Cellobiohydrolase activity was analyzed in leaves incubated during 55 days. Note that in panel A points indicate decomposition rates and vertical bars indicate the standard error of the regression. Different letters indicate significant differences on dependent variables in each panel based on ANCOVA (A) and ANOVA (B) analysis followed by Tukey-t test, respectively. Dotted lines indicated the interval confidence of the regression (95%).

Leaf litter *k* expressed in terms of daily sum of water velocity (i.e., velocity standardized-*k*, in velocity-days^{-1}) significantly fitted to the exponential decay model ($0.82 < r^2 > 0.96$, $p < 0.0001$) at all sampling locations (Table S5.2.; see annexes section). Velocity standardized-*k* also differed among sampling locations (ANCOVA, $p < 0.01$), and values were negative related with water velocity at each location (Figure 5.6.).

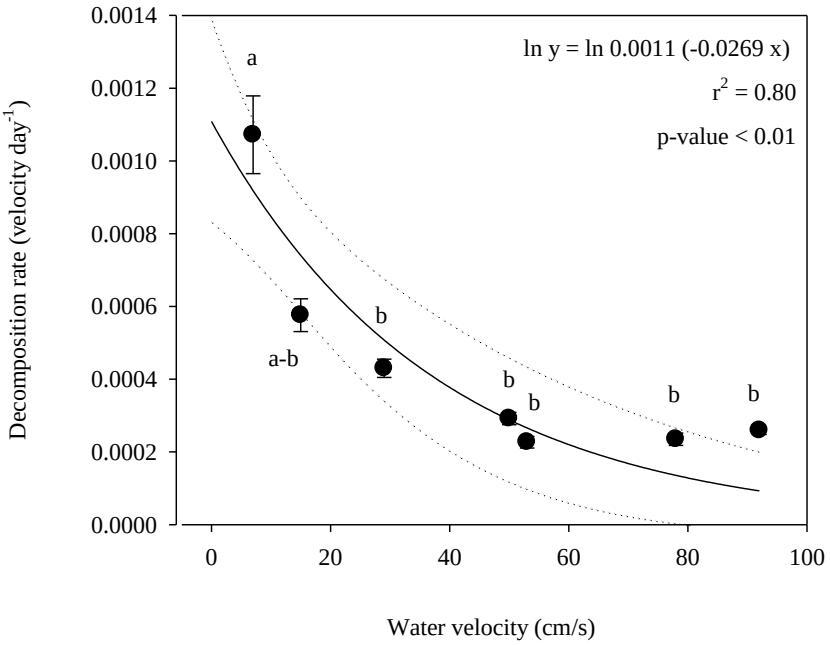

Figure 5.6. Exponential relationship between velocity standardized-*k* and water velocity. Vertical bars indicate the standard error of the regression. Different letters indicate significant differences on decomposition rates on ANCOVA analysis followed by Tukey-t test. Dotted lines indicated the interval confidence of the regression (95%).

Extracellular enzyme activity of CBH in leaf litter measured after 55 days of incubation ranged from 8.1 – 61.6 µmol MUF g DM^{-1} h^{-1}. The CBH activity significantly differed among sampling locations (ANOVA, $p < 0.01$), and decreased with increased water velocity ($r^2 = 0.27$, $p < 0.01$; Figure 5.5. B).

5.5. Discussion

The capacity of stream ecosystems to decompose leaf litter inputs has been traditionally addressed by quantifying leaf litter decomposition rates (*k*) in several habitats within the stream, and up-scaling the average of the obtained *k* values at ecosystem level (Webster and Benfield, 1986; Woodward et al. 2012). Therefore, the examination of leaf litter decomposition is beset with uncertainty because these studies provided little or no information regardless of how *k* can vary within a reach. Our results revealed that leaf litter decomposition can remarkably vary within a stream reach, and that this variability can be driven by the spatial heterogeneity of water velocity in the reach. Our results also demonstrate that the spatial variability of water velocity within a reach can influence the capacity of the reach to retain the inputs of leaf litter as well as the spatial distribution of these inputs in the reach. Therefore, our results eventually extent the influence of sub-reach scale variability of water velocity on in-stream processes (Peipoch et al. 2016) by further suggesting a relevant effect on leaf litter dynamics.

Influence of water velocity on retention and spatial distribution of leaf litter inputs

At base flow, the retention of leaf litter inputs has commonly been assumed as a static process; and thus, the spatial re-distribution of retained leaves within a reach has been

associated with events of increasing discharge (i.e., floods; Webster et al. 1994; Wallace et al. 1995). However, our results show that retained leaves in a reach can be spatially re-distributed over time and travel longer distances even under stable discharge conditions. Therefore, we suggest that leaf litter retention, distribution, and transport in streams are dynamic processes even under base flow conditions. This behavior has been usually not considered in previous studies using a single period of time between leave addition and collection (Larrañaga et al. 2003; Cordova et al. 2008). Therefore, some assumptions provided by these studies should be reconsidered. For instance, most previous studies indicated that the average distance travelled by leaves might be a good predictor of their final spatial distribution within the stream. These studies also suggest that leaves become retained close to the input site; and thus, generally travel short distances (Snaddon et al. 1992, Raikow et al. 1995, Dewson et al. 2007). Our results support this idea, but only when we focus on short-time periods (i.e., few hours) after leaf litter inputs. As time since leaf litter inputs increases, the spatial heterogeneity of water velocity within the reach becomes a more important factor describing the spatial distribution of leaf litter. In fact, leaves re-distribute themselves along the reach, with a transition from high to low velocity zones. This suggests that low velocity zones favors leaf litter retention (Hoover et al. 2006). Alternatively, the accumulation of leaves at low velocity zones could be explained by the higher dominance of low velocity zones within the reach (~70%, Table S1). Nevertheless, we found that under mid-velocity conditions leaves were more effectively retained than expected from the relative abundance of locations within the reach. Therefore, at mid-velocity conditions leaves can also be effectively retained and exposed to decomposition by microbial assemblages. We do not know the underlying mechanism responsible for this unexpected result, but it could be that under this velocity range the forces retaining

leaves to streambed obstacles could be higher than those promoting leaf litter re-suspension. Previous studies conducted in headwaters streams observed that leaves can be trapped in riffles due to the presence of streambed obstacles (i.e., cobbles, rocks, wood) where leaves can be easily attached (Speaker et al. 1984, Hoover et al. 2010). This fact might be especially relevant in headwater streams due to the dominance of large alluvial substrata.

Influence of water velocity on decomposition rates and microbial activity of leaf litter

The values of *k* measured in this study for alder leaf litter varied ~3 fold within the study reach and this spatial variation was explained by variation in water velocity. These results indicate that water velocity is an important factor influencing leaf litter decomposition within the reach. The range of *k* values for alder (i.e., 0.0076 d^{-1} to 0.0222 d^{-1}) is comparable with the range of *k* values reported for several streams (Webster and Benfield 1986; Woodward et al. 2012; Bastias et al.2017) and among different leaf litter species (Webster and Benfield, 1986). Therefore, the variability of leaf litter *k* provided in the literature could have been maximized or even counterbalanced depending of the particular velocity conditions during the decomposition process (Woodward et al. 2012; Bastias et al. 2017). Hence, the interaction of water velocity with factors controlling leaf litter decomposition such as water temperature and water column nutrient concentrations should be considered if we aim to understand how leaf litter decomposition occurs under different environmental conditions. In this regard, we expected that differences in *k* values should be mainly explained by physical processes, therefore if all sampling locations within the reach had similar environmental conditions we expected that the influence of the activity of

microbial decomposers to be similar. In fact, during the experiment, temperature, nutrient concentrations, and oxygen concentration were relatively similar within the reach. Therefore, the positive relationship between water velocity and *k* observed in this study could be explained by leaf litter fragmentation, supporting results from previous studies (Ferreira et al. 2006). However, our results revealed that the effect of water velocity on leaf litter decomposition increases as incubation time proceeds. This may be likely explained by the fact that the toughness of the leaves may buffer the effect of water velocity during the early stages of decomposition. The reduction of leave toughness throughout decomposition has been previously observed and has been mainly attributed to the conditioning and development of microbial assemblages on leaf litter (Quinn et al. 2000; Artigas et al. 2011). Therefore, the effect of physical abrasion by water velocity could be subjected to the stage at which microbial assemblages are developed on the leaf litter surfaces, which may explain the observed increase of physical fragmentation during decomposition process (Carton and Martinson 1990). This is in agreement with previous studies, which suggest that leaf litter decomposition is initially driven by leaf litter leaching and microbial colonization, and then, by the mechanical effect of physical abrasion and macroinvertebrate activity (Webster and Benfield 1986).

To further explore how water velocity influences leaf litter decomposition, we standardized decomposition rates by water velocity using a similar approach as that used to standardize *k* values among sites by water temperature (i.e., *k* values per unit of degree days). We expected that *k* standardized by water velocity should remain constant among sampling locations if physical abrasion was the main driver explaining the observed spatial variability of *k* within the reach. However, standardized-*k* by water velocity still differed among sampling locations, but, unexpectedly, higher values

coincided with low velocity conditions. These results suggest that the influence of water velocity on leaf litter decomposition can go beyond physical fragmentation and can also be explained by biological degradation. In fact, results from the CBH activity support this suggestion since the capacity of microbial decomposers to degrade cellulose polymeric compounds (i.e., CBH activity) was higher under low water velocity conditions, and it sharply decreased with increasing water velocity. Nevertheless, our study does not allow explaining the causes of the negative influence of water velocity on microbial enzymatic activity, but suggest that higher water velocity did not enhance the capacity of microbes to degrade the leaf litter matter. In this regard, previous studies contrast with our findings, showing a positive influence of water velocity on several parameters associated with microbial decomposers, such as fungal sporulation rates and cumulative conidial production (Ferreira et al. 2006), but not in the microbial capacity to produce enzymatic activities. Probably, the amount of energy invested to degrade leaf litter polymeric compounds is higher under low velocity environments due to the reduction of water turbulence and physical abrasion over leaf litter surfaces. In this sense, the influence of water velocity on microbial development and activity should be examined in detail to better understand the biological role on in-stream leaf litter dynamics.

Influence of water velocity on leaf litter dynamics at reach scale

Stream discharge is a pivotal driver of leaf litter retention in streams, which can further dictates whether leaf litter inputs can act as an effective source of energy and matter for microbial communities of receiving stream reaches. At low stream discharge inputs of leaf litter are barely transported downstream, whereas at high stream discharge leaf litter

inputs are basically exported (Larrañaga et al. 2003; Cordova et al. 2008). However, at intermediated discharge, stream flow strongly interacts with streambed structure generating a complex physical template that drives spatial variation in water velocity, which can influence leaf litter retention (Richarson et al. 2009). Thus, the complexity of streambed has been documented as a relevant factor determining the standing stocks of leaf litter within the reach. To date, it has been difficult to determine a reliable parameter to quantify the complexity of streambed structure and how it may influence leaf litter retention within the stream. In this sense, this study sheds some light on the mechanisms driving this uncertainty and suggests that the heterogeneity of water velocity at reach scale is a relevant factor to understand the retention and spatial distribution of leaf litter inputs, especially under intermediate flow conditions. High variability of water velocity within the reach may increase the probability that leaf inputs will be retained and decomposed by generating a complex set of suitable habitats. In addition, our results suggest that the spatial distribution of water velocities within the reach may also dictate the rates of leaf litter decomposition as well as the main process involved in leaf litter mass loss. On one hand, our results increase the certainty that leaves retained under high-velocity habitats (i.e., > 50 cm s^{-1}) may be more easily re-suspended and further exported downstream. Moreover, physical fragmentation in these environments may increase *k* ~270-to-292% in comparison to that found in low-velocity locations. Thus, an increase of the relative proportion of sites covering this velocity range within the reach could increase the export of leaves both, as coarse particles and as fine particles after leaves are physically fragmented. On the other hand, in stream reaches with a high proportion of sites with relatively fast velocities (i.e., ranging from 20 to 50 cm s^{-1}) leaf inputs will be effectively retained, but will undergo physical fragmentation. In fact, under these conditions leaf litter decomposition may even

increase by 39-to-49% in comparison to that measured at low-velocity sites. Therefore, in this case, leaf litter inputs will be partially fragmented and exported to downstream sites, without major contribution to the metabolism of recipient reach. Finally, in stream reaches dominated by sites with low velocity, leaves will be easily retained and biologically metabolized. Considering all results together, this study indicates that the relevance of leaf litter inputs as organic matter source to in-stream communities can be subjected to the hydro-morphological characteristics of the receiving stream reaches, since they determine the spatial heterogeneity of water velocity within the reach. In addition we found that water velocity is a factor controlling different aspects of leaf litter dynamics in streams because not only influences the fraction of leaf litter retained but also how these inputs are spatially distributed and further processed within the streams. Therefore, this factor should be considered in future studies to increase our understanding of how leaf litter inputs can effectively fuel the metabolism of stream ecosystems.

CHAPTER 6: CHEMICAL AND OPTICAL PROPERTIES OF DIFFERENT LITTER LEACHATES INFLUENCE IN-STREAM NUTRIENT POOL AND MICROBIAL ACTIVITY

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6.1. Abstract

We studied how chemical and optical properties of the leachates vary among different coarse particulate organic matter (CPOM) sources, and how such potential variation can influence the activity of microbial assemblages in streams. We produced leachates from 6 leaf litter riparian tree species, and from a mixture of fruits and of twigs from these species. For each type of CPOM leachate, we analyzed the concentration of dissolved organic carbon (DOC) and organic and inorganic nitrogen (N) and phosphorus (P) forms. We also analyzed optical indexes associated with the degree of aromaticity of the dissolved organic matter (DOM) of leachates, such as $SUVA_{254}$, E_2/E_3 and E_4/E_6. Additionally, we estimated rates of microbial metabolic activity associated with each leachate type using the Resazurin (Raz) - Resorufin (Rru) system under laboratory conditions. Results show that leachates from riparian CPOM are sources of high-quality DOC, dissolved organic N and dissolved inorganic P. In addition, Rru production rates were positively related to the degree of aromaticity and the NO_3^- concentrations of leachates. Together these results suggest that the management of riparian vegetation could have significant implications for the DOC and nutrient dynamics as well as for the heterotrophic activity of stream ecosystems.

6.2. The role of leaf litter leachates in stream ecosystems

In forested headwater streams, inputs of coarse particulate organic matter (CPOM) from riparian zones (i.e., leaf litter, fruits and twigs) undergo an initial loss of mass due to the leaching of elemental constituents. CPOM inputs are used as colonizing substrate as well as source of carbon (C) and nutrients (i.e., nitrogen and phosphorous) for in-stream microbial communities. CPOM is also mechanically fragmented by macroinvertebrates and physical abrasion (Webster and Benfield, 1986). Therefore, CPOM inputs can act as the primary energy source for the metabolism of these ecosystems (Fisher and Likens 1973, Vannote et al. 1980) as well as, can influence in-stream nutrient cycling (Hall and Meyer 1998, Valett et al. 2008) and food web composition and function (Webster et al. 2000).

During the initial phase (i.e., ~24 h), leaching of dissolved organic matter (DOM) from CPOM can constitute an important energy source to in-stream microbial activity (Webster and Benfield, 1986). Some studies have shown that leachates from CPOM can contribute approximately up to 30-42% of the total dissolved organic carbon (DOC) pool in streams during autumn (McDowell and Fisher 1976, Meyer et al. 1998). In addition, leachates from CPOM also contain dissolved inorganic nitrogen (DIN) forms, such as nitrate (NO_3^-) and ammonium (NH_4^+), and soluble reactive phosphorous (SRP) (Wymore et al. 2015). Thus, leachates from direct inputs of riparian CPOM have the potential to influence the in-stream dynamics of the dissolved organic and inorganic elemental pool, which may further affect the activity of microbial assemblages of these ecosystems and overall temporal dynamics of ecosystem metabolism.

The bioavailability of leachates from riparian CPOM inputs to in-stream microbial assemblages is related to the amount of elements released. As an example, Wymore et

al. (2015) suggested that variation in dissolved N concentrations of leachates among types of CPOM could result in differences in in-stream microbial activity. Furthermore, the bioavailability of leachates is related to the quality of the DOM. In fact, low-molecular weight amino acids and carbohydrates (i.e., compounds with low degree of aromaticity) are rapidly mineralized (Amon et al. 2001, Balcarczyk et al. 2009), whereas humic-like compounds with higher molecular weights (i.e., higher aromaticity) tend to be less bioavailable and, thus, have longer residence times in the water column (Fellman et al. 2009). Despite differences in DOM quality among CPOM inputs, the bioavailability of DOC in streams is also influenced by the origins of its inputs (Meyer et al. 1987, Fellman et al. 2009). DOC entering into streams via terrestrial runoff (from plants and soils) is previously processed by soil microbial communities. Thus, this DOC is usually considered more recalcitrant for in-stream heterotrophic communities than other sources of DOC that mediate the microbial activity in streams (Tranvik 1988, McKnight et al. 2001). In contrast, leachates from plant litter input may provide streams with fresh DOC and nutrient resources, which could strongly influence in-stream microbial heterotrophic activity. However, despite this potential influence of CPOM leachates on in-stream microbial activity, information on how leachate characteristics vary among different sources of riparian species, and how such potential variation can influence their effect on the in-stream microbial activity, is scarce (but see Wymore et al. 2015). Assessment of DOM quality of the leachates has been difficult, but several proxies and indexes based on fluorescence spectroscopy have recently been developed and used to infer the potential bioavailability of DOM from freshwaters (Murphy et al. 2010, Cory et al. 2011). For instance, the specific ultraviolet absorbance at 254 nm (i.e., $SUVA_{254}$) is being widely used as a proxy of the degree of humification, aromaticity, and molecular weight of DOM (McKnight et al. 2001, Weishaar et al. 2003). In fact,

Weishaar et al. (2003) reported a positive correlation between $SUVA_{254}$ and aromaticity of DOM from leachates determined by ^{13}C nuclear magnetic resonance (NMR). Other recently used indexes associated with DOM aromaticity are the ratios E_2/E_3 (Wang et al. 2009, Leeben et al. 2010) and E_4/E_6 (Peuravuori and Pihlaja 1997, Fuentes et al. 2006). The E_2/E_3 is the ratio between the specific absorbance at 250 nm and that at 365 nm, and it is inversely correlated with DOM aromaticity (Peuravuori and Pihlaja 1997, McDonald 2004). The E_4/E_6 is the ratio between the absorbance at 465 and that at 665 nm, and it is positively correlated with DOM aromaticity. This ratio has been predominantly used in soils.

In this study, we explore (i) how chemical and optical properties of the leachates vary among different CPOM sources, and (ii) how such potential variation can influence the activity of microbial assemblages in streams. We expected that leachates from different CPOM sources will have different chemical and optical properties. We hypothesized that a high degree of aromaticity among CPOM leachates (i.e., higher values of $SUVA_{254}$ and E_4/E_6 ratios and lower values of E_2/E_3 ratios) would be negatively related to microbial activity (expressed as Rru production), because highly aromatic compounds are more difficult to degrade. We also hypothesized that CPOM leachates with higher concentrations of dissolved nutrients (i.e., N and P forms) would result in higher microbial activity if the activity of microbial assemblages is nutrient limited (Kroer 1993, Zweifel et al. 1993).

6.3. Methods

Production of leachates from different riparian CPOM sources

We collected leaf litter from 6 tree species that are broadly distributed in riparian zones of the Mediterranean region, i.e. alder (*Alnus glutinosa*), black poplar (*Populous nigra*), black locust (*Robinia pseudoacacia*), ash (*Fraxinus excelsior*), sycamore (*Platanus x hispanica*), and holm oak (*Quercus Ilex*). We also collected fruits and twigs from these tree species. Samples of these different CPOM sources were collected in Font del Regàs stream, a 3rd order Mediterranean stream draining La Tordera catchment (NE Spain; 41º50´N, 2º30´E, 300 m a.s.l). Samples of riparian CPOM sources were collected with aerial traps (n = 5) made by a polyvinyl chloride frame (PCV, 1 m^2) and a plastic mesh (5 mm mesh size). Traps were fastened to the riparian trees adjacent to the stream and hung over the stream channel along a 100-m reach. We collected all the CPOM material accumulated in the traps during peak senescence (from mid-October to early November, 2013). The samples were transported to the laboratory in paper envelopes, and were air dried at room temperature (20 ºC, 30% moisture) for 24 h. Approximately 1 g of each CPOM type (5 replicates) was placed in a 120-ml plastic tubes with 100 ml of deionized water. Then, samples were placed in a shaker during 24 h (20 ºC at 75 rpm) to facilitate the extraction of the leachates. After the 24 h extraction, we filtered the leachates through ashed (500 ºC for 5 h) FVF glass filters (0.7 µm pore size) to exclude small particles. The leachates were analyzed for chemistry (10 ml) and optical properties of DOM (10 ml). Simultaneously, 50 ml of leachates were used for the incubations with Raz-Rru metabolic system (see below).

Measurements of chemical and optical properties of leachates

For each leachate sample, we analyzed the concentration of DOC and total dissolved N (TDN) by high-temperature catalytic oxidation on a Shimadzu TOC-VCSH + TNM-1 + ASI-V analyser. Leachate concentration of total phosphorus (TP) was analysed by acid-hydrolysis and measured with colorimetric methods. We measured the concentrations of $NO_3^- + NO_2^-$ (NO_3^-; Cd-Cu reduction), NH_4^+ (phenate method), and SRP (molybdate blue) of leachates by Continuous Flow Analysis (CFA) with a Bran+Luebbe auto-analyser. We calculated dissolved organic nitrogen (DON) as TDN minus the sum of NO_3^-, NO_2^-, and NH_4^+ and dissolved organic phosphorous (DOP) as TP minus SRP. All chemical analyses were conducted at the Nutrient Analysis Service of the ICM-CSIC (Barcelona). Concentration of different solutes was multiplied by the water volume used in the leachate production and divided by the dry mass (DM) of the CPOM used to obtain the leachates. Therefore, chemical characterization of solute concentration of different CPOM leachates is expressed in µg or mg of solute per g DM^{-1}.

The leachates were also characterized for optical indexes associated with their degree of aromaticity. In this sense, we divided the specific absorbance at 254 nm by the DOC concentration (mg L^{-1}) to estimate $SUVA_{254}$ (in units of L mg^{-1} C m^{-1}) (McKnight et al. 2001), the ratio of the specific absorbance at 250 nm and that at 365 nm to estimate the E_2/E_3 (McDonald, 2004), and the ratio of the specific absorbance at 465 nm and that at 665 nm to estimate the E_4/E_6.

Estimation of microbial activity associated with CPOM leachates

The effect of leachates from different leaf litter sources on in-stream heterotrophic activity has been assessed in laboratory incubations by quantifying the rates of

dissolved oxygen (DO) consumption (Wymore et al. 2015). In the present study, we used a novel approach to measure responses of microbial metabolic activity to leachates of different riparian CPOM types: the Resazurin (Raz) - Resorufin (Rru) chemical system. The reduction of Raz to Rru has been used in previous studies as a good tracer to infer microbial metabolic activity of specific in-stream biotic components (O'Brien et al. 2000, Guerin et al. 2001, McNicholl et al. 2007) and metabolism at whole-reach scale (Haggerty et al. 2009). Microbial activity based on these measurements is not subjected to limitations of dissolved oxygen gas exchange and it is a good tool to compare microbial activity among different CPOM treatments. In Font del Regàs stream, we collected fine benthic organic matter (FBOM) as the source of microbial assemblages to estimate microbial activity associated with the different types of CPOM leachates. The upper layer (~first 2 cm) of the streambed sediment was gently stirred to re-suspend the FBOM, which was then collected with a syringe (100 ml) following the procedure described by von Schiller et al. (2009). In the laboratory, we incubated 50 ml of the leachates from each CPOM type with 100 µL of homogenized slurry of FBOM (5 replicates per CPOM leachate type). We then added 10 ml of Raz standing stock solution, which resulted in a target initial Raz concentration of 200 µg L^{-1} in the incubations. We collected 5 mL samples from each incubation vial (8 CPOM leachate types and 5 replicates per leachate type) every 30 minutes during 4 hours. Fluorescence of collected samples was measured at 571 and 585 nm of excitation and emission wavelengths, respectively, to estimate Rru concentration using a spectrofluorophotometer (Shimadzu/ RF-5000) (Haggerty et al. 2008). We calculated Rru production rates as the difference in Rru concentration between samples at time 0 minutes and at ~1.2 h of incubation, because this incubation timeframe showed a linear increase of Rru concentration consistently among all the incubations. Results of the Rru

production rates were expressed as mmol of Rru produced per incubation time (h) and CPOM dry mass (g) used to generate each leachate.

Statistical analysis

To examine differences in the chemical and optical parameters of the leachates among the different CPOM types, we used a one-way analysis of variance (ANOVA) with CPOM type as fixed factor followed by Tukey´s post hoc-test for each variable examined. We also calculated the coefficient of variation (CV) for each chemical and optical variable considering data from all CPOM types together, to assess the degree of variability for each variable associated with the different CPOM types. Additionally, we conducted a PCA analysis considering all chemical and optical variables of the leachates to evaluate relationships among them, and to assess which variables mostly contributed to the observed variability among leachates of the different CPOM types.

We also used one-way ANOVA to determine differences in Rru production rates associated with leachates from the different CPOM types, with CPOM type as fixed factor, followed by Tukey´s post hoc-test. We used partial least square (PLS) regressions to explore how Rru production rates from different leachates were related to the chemical and optical properties of the leachates. PLS regression is a linear multivariate model, which produces latent variables (PLS components) extracted from predictor variables that maximize the explained variance of the dependent variable. PLS regression is especially useful when predictor variables are correlated (Carrascal et al. 2009). The evaluation of the PLS regression models was based on the level of variance explained (r^2), loadings of the independent variables, and the variable influence on projection (i.e., VIP). The independent variable loading describes the relative strength and direction of the relationship between independent (i.e., chemical and optical

characteristics) and response variable (i.e., Rru production rates). The VIP value summarizes the importance of each predictor variable. The limit for a variable to be included in the final model was a VIP value of 1. Finally, we examined pairwise linear regression analyses between Rru production rates and those variables that were found to be significant predictors in the PLS analysis. If necessary, variables were log-transformed to meet the requirements of parametric tests (ANOVA and linear regression), but PLS regression does not assume normally distributed data. PLS regression analyses were performed in R version 3.2.4 (R Core Team 2012) using the PLS package version 2.5-0 for the PLS models (Mevik et al. 2011).

6.4. Results

Chemical and optical properties of the leachates

DOC concentration in the leachates differed among CPOM types (ANOVA, $p < 0.001$) with the lowest values in leachates from fruits and twigs and the highest in those from leaf litter of holm oak (Table 6.1.). NO_3^- concentration also differed among CPOM types (ANOVA, $p < 0.001$), and was highest in leachates from leaf litter of alder (Table 6.1.). Contrastingly, NH_4 concentration was highest in leachates from fruits and black locust leaf litter (ANOVA, $p < 0.001$). DON concentration also differed among CPOM types and was the highest in leachates from black locust leaf litter (ANOVA, $p < 0.001$; Table 6.1.). Concentration of SRP in leachates was quite similar among leaf litter types, but showed significantly lower values in leachates from fruits and twigs (ANOVA, $p < 0.001$). Likewise, DOP concentration was similar among CPOM types (ANOVA, $p > 0.05$) (Table 6.1.). For the measures of aromaticity, values of $SUVA_{254}$ differed among CPOM types (ANOVA, $p < 0.001$) with leachates from alder leaf litter showing the highest values (Table 6.1.). The E_2/E_3 differed among CPOM types (ANOVA, $p < 0.01$) and was the lowest in leachates from alder. E_4/E_6 index also varied among CPOM types (ANOVA , $p < 0.001$) showing the highest values in leachates from alder, black poplar, black locust, and ash leaf litter.

Table 6.1. Mean (± 1 SE) values of chemical and optical variables from leachates produced by leaf litter, fruits and twigs from different riparian tree species types of allochthonous coarse particulate organic matter (CPOM) after 24-h incubation in distilled water. Data are presented as mass of C, N, and P per g of CPOM dry mass except $SUVA_{254}$ (L mg C^{-1} m^{-1}). Different letters indicate significant differences at $p = 0.05$. DOC: dissolved organic carbon; DON: dissolved organic nitrogen; DOP: dissolved organic phosphorous and SRP: soluble reactive phosphorous.

Liter material types	DOC (mg C g^{-1})	NO_3 (µg N g^{-1})	NH_4 (µg N g^{-1})	DON (µg N g^{-1})	PO_4 (µg P g^{-1})	DOP (µg P g^{-1})	$SUVA_{254}$	E_2/E_3	E_4/E_6
Alder (AL)	41.1 (1)^{b}	20.5 (3)^{a}	8.4 (3)^{c}	583 (47)^{b}	276.0 (22)^{a}	62.9 (4)^{a}	0.15 (0.02)^{a}	0.16 (0.01)^{a}	13.7 (2)^{a}
Black Poplar (PO)	48.4 (3)^{b}	4.6 (1)^{b}	1.6 (1)^{c}	223 (49)^{d}	191.0 (31)^{ab}	53.3 (11)^{a}	0.08 (0.01)^{bc}	0.43 (0.02)^{b}	9.5 (2)^{abc}
Black Locust (LO)	45.6 (2)^{b}	5.4 (0.4)^{b}	114.0 (16)^{b}	1031 (43)^{a}	253.0 (19)^{a}	82.6 (6)^{a}	0.08 (0.01)^{b}	0.33 (0.03)^{ab}	12.5 (2)^{ab}
Ash (AS)	57.1 (7)^{ab}	4.4 (0.4)^{b}	2.7 (0.2)^{c}	420 (63)^{c}	298.0 (38)^{a}	84.2 (21)^{a}	0.07 (0.01)^{bc}	0.40 (0.02)^{b}	10.7 (0.5)^{abc}
Sycamore (SY)	47.9 (3)^{b}	1.3 (0.2)^{b}	2.4 (1)^{c}	116 (9)^{d}	271.0 (53)^{a}	103 (36)^{a}	0.02 (0.005)^{d}	0.52 (0.02)^{b}	4.8 (1)^{c}
Holm Oak (OA)	64.2 (5)^{a}	1.7 (0.6)^{b}	3.7 (3)^{c}	106 (17)^{d}	199.0 (8)^{ab}	43.9 (5)^{a}	0.03 (0.005)^{cd}	0.49 (0.02)^{b}	4.9 (0.7)^{c}
Fruits (FR)	21.7 (4)^{c}	2.7 (0.3)^{b}	180.0 (36)^{a}	132 (18)^{d}	95.3 (26)^{bc}	64.2 (41)^{a}	0.04 (0.01)^{bcd}	0.42 (0.09)^{b}	6.6 (0.3)^{bc}
Twigs (TW)	25.7 (1)^{c}	2.5 (0.4)^{b}	9.4 (8)^{c}	136 (24)^{d}	64.7 (24)^{c}	18.4 (5)^{a}	0.02 (0.005)^{d}	0.39 (0.1)^{b}	6.0 (1)^{c}
CV (%)	34.5	139.1	175.6	92.6	49.8	77.6	72.4	37.6	47.0

Considering data from all CPOM types, the highest range of variability based on the coefficient of variation was observed for NH_4, NO_3 and DON concentrations, and the lowest was observed for concentrations of DOC and SRP and the optical indexes E_2/E_3 and E_4/E_6 (Table 6.1.).

Results from the PCA revealed that 40% of the variability among study cases (i.e., CPOM leachate types) was explained by component 1, which was mostly loaded by NO_3^- and DON concentrations, and the optical indexes. Component 2 explained 22% of the variability among study cases and concentrations of SRP, DOP, DOC, and NH_4^+ had a major load on this component. Results from the PCA also revealed that $SUVA_{254}$ was positively correlated with NO_3 and the E_4/E_6 index and negatively correlated with the E_2/E_3 index (Figure 6.1.). Hence, a higher degree of DOM aromaticity in the leachates of the different study CPOM types is accompanied by a higher concentration of NO_3. Lastly, concentrations of SRP in leachates was positively correlated with concentrations of DOP and DOC and negatively correlated with concentrations of NH_4 (Figure 6.1.).

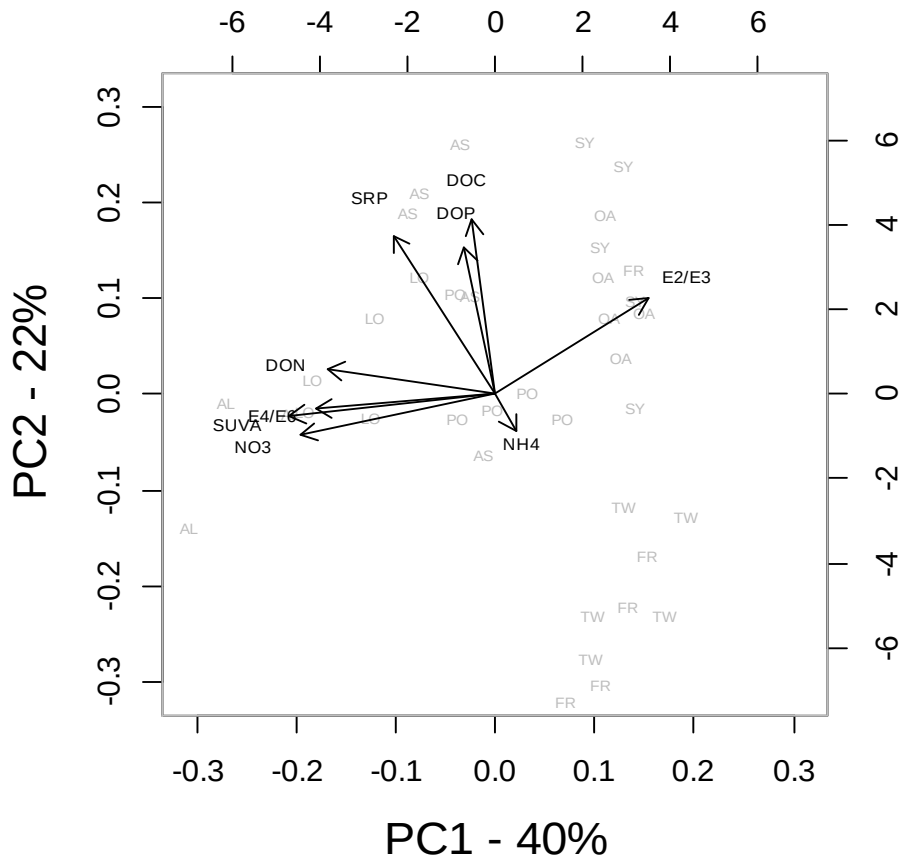

Figure 6.1. Principal component analysis (PCA) considering data from chemical and optical variables associated with leachates. PC1 and PC2 explain 40% and 22%, respectively, of the variability among variables. AL (alder), PO (black poplar), LO (black locust), SY (sycamore), OA (holm oak), FR (fruits), and TW (twigs). DOC: dissolved organic carbon; DON: dissolved organic nitrogen; DOP: dissolved organic phosphorous, and SRP: soluble reactive phosphorous.

Estimation of metabolic activity associated with leachates

Rru production rates associated with the leachates differed among CPOM types (ANOVA, $p < 0.001$) and ranged from 0.65 to 5.20 mmol Rru g DM^{-1} min^{-1}. Higher Rru production rates were observed in leachates from alder leaf litter, and lower values were observed in leachates from sycamore leaf litter, fruits, and twigs (Figure 6.2.).

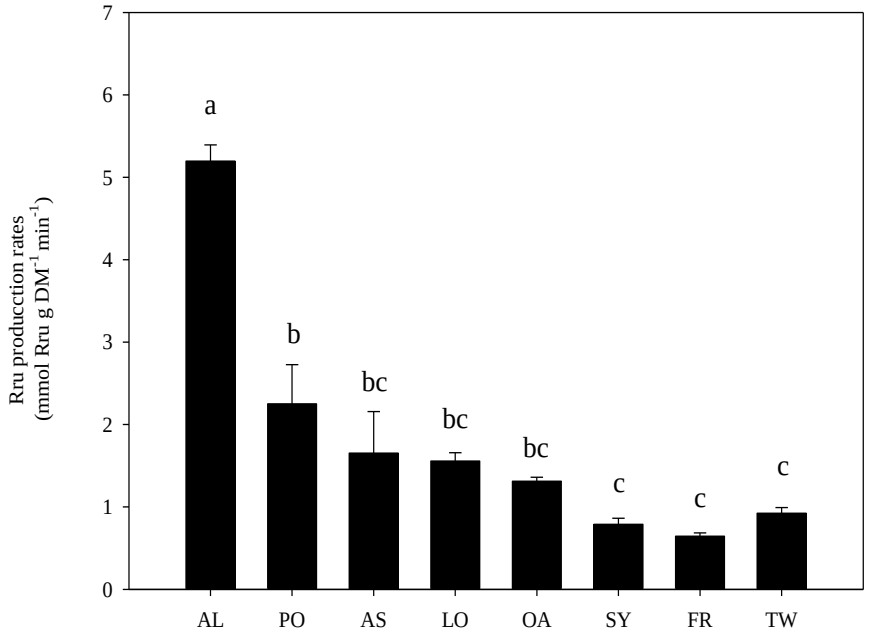

Figure 6.2. Rru production rates used as a proxy of microbial activity respiration measured from different leachates among CPOM materials analyzed. Different letters indicate significant differences ($p < 0.05$) in Rru production rates among CPOM materials. AL (alder), PO (black poplar), LO (black locust), SY (sycamore), OA (holm oak), FR (fruits), and TW (twigs).

The PLS regression analysis showed that variation in Rru production rates was best explained by the combination of NO_3 concentration and the optical variables of leachates (Figure 6.3.).

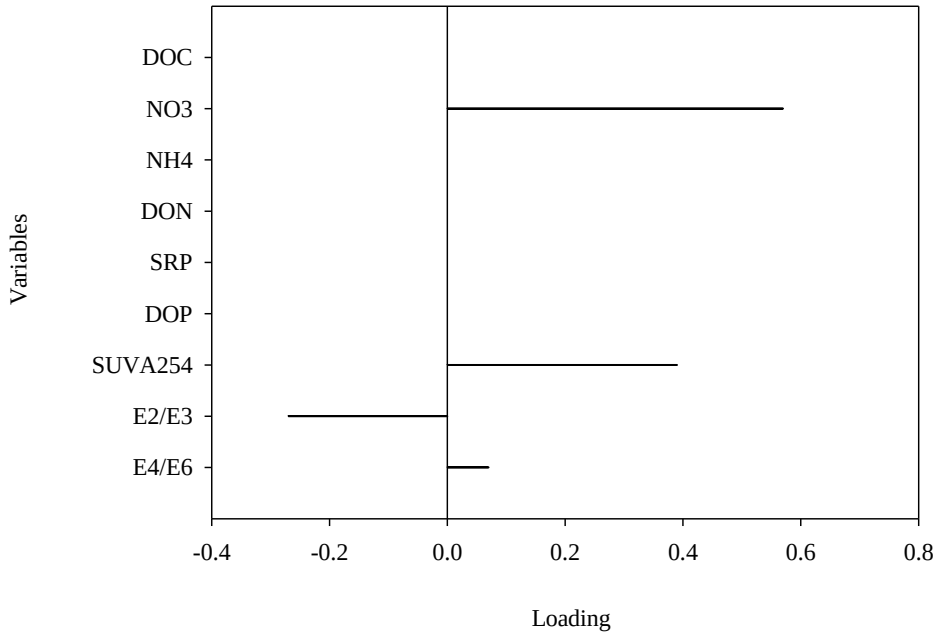

Figure 6.3. Results from partial least square (PLS) regressions on Rru production rates and both chemical and optical variables associated with leachates. Variance explained was 80.9 % (two components). Only variables with a VIP ≥ 1 are considered as predictor variables.

Rru production rates were positively related to NO_3 concentration, $SUVA_{254}$ and E_4/E_6 index, and negatively related to E_2/E_3 index (Figure 6.4.).

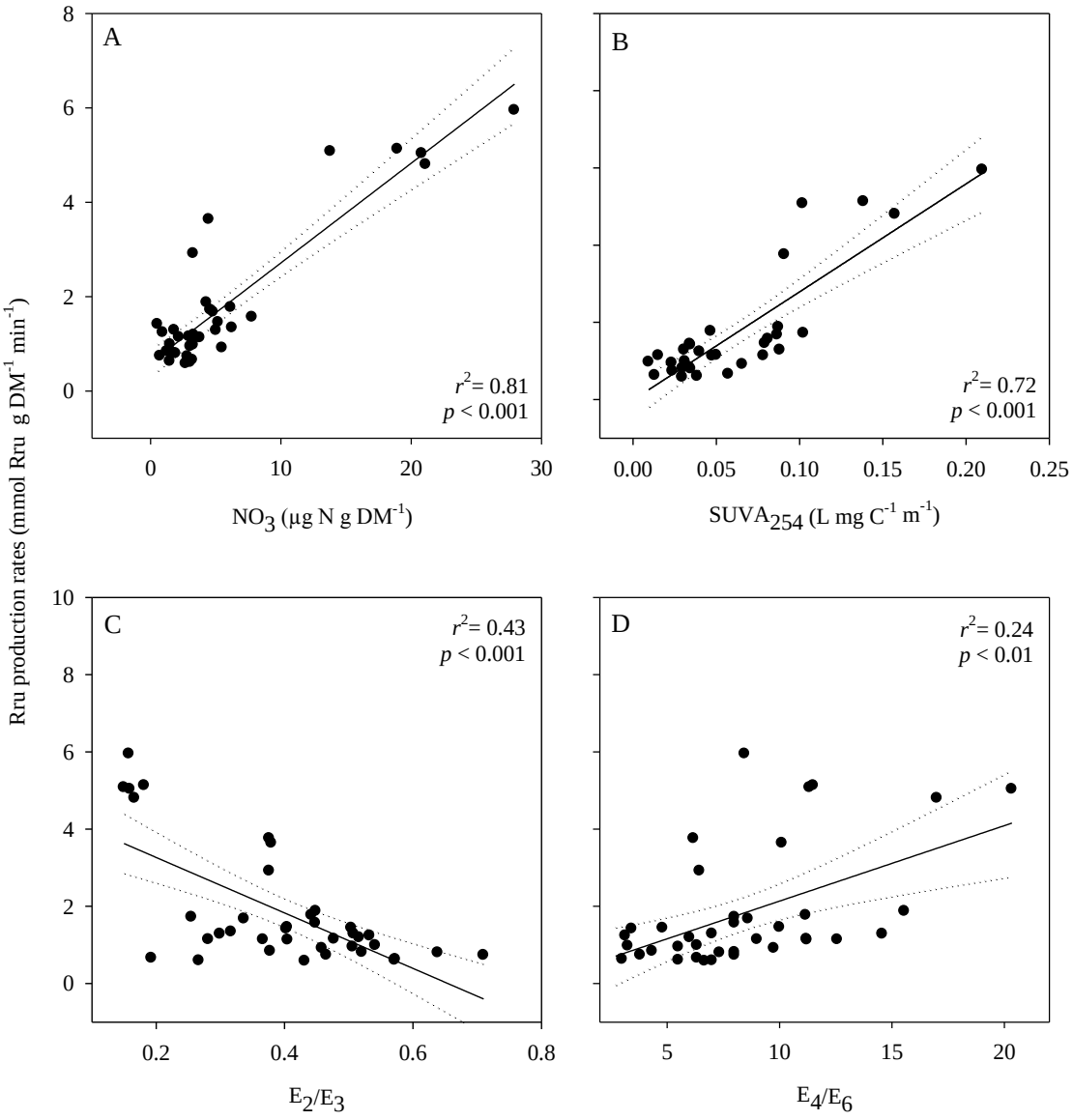

Figure 6.4. Linear regressions between Rru production rates and the significant predictor variables obtained from PLS regression analysis. Coefficients of the linear regressions (r^2) and *p*-values (*p*) are shown for each variable.

6.5. Discussion

The importance of leachates from riparian CPOM inputs to in-stream DOM, and N and P pools

Our study shows that leachates directly released from riparian CPOM inputs may constitute a relevant DOM source to streams (Meyer and Wallace, 1998). In fact, DOM from leachates has lower $SUVA_{254}$ values (0.02-0.15 L mg C^{-1} m^{-1}) compared to values reported for other allocthonous DOM sources from riparian soils (1.5–4.7 L mg C^{-1} m^{-1}; Wickland et al. 2007, Balcarczyk et al. 2009). This finding is in agreement with previous results suggesting that leachates from riparian CPOM inputs constitute a higher quality DOM source to streams (i.e., less aromatic and recalcitrant) than other DOM sources from terrestrial origins such as groundwater, terrestrial runoff, and snowmelt (Allan and Castillo, 2008; Wymore et al. 2015). One reason why leachates contain high-quality DOM may be that the CPOM that they originate from enters into streams directly (McDowell and Fisher, 1976), without being processed by microbial soil communities. Our results additionally indicate that leachates from riparian CPOM inputs can act as relevant sources of dissolved organic and inorganic N and P to streams, supporting previous results (e.g., Webster and Benfield 1986, Wymore et al. 2015); and thus, they can influence the in-stream dynamics of dissolved N and P, especially during the leaf fall period. However, we also found that the influence of leachates on the relative proportion of dissolved inorganic and organic stream pools varies depending on the element considered (i.e., N and P). In particular, DON was the dominant form of dissolved N in leachates regardless of the CPOM type (~94% from the total dissolved N). This is in agreement with results from previous studies (Wymore et al. 2005) and indicates that, in terms of dissolved N, inputs of riparian CPOM mostly

provide streams with DON. In contrast, the inorganic form of P accounted for ~78% of the total dissolved P in leachates, suggesting that CPOM inputs can be important sources of dissolved inorganic P to streams.

Overall, these results suggest that CPOM inputs are not only relevant sources of particulate C and nutrients to microbial and macroinvertebrate decomposers in streams, but they can also contribute to the dissolved C, N, and P pools via leaching of the CPOM inputs. The relevance of CPOM leachates to streams may be maximized in forested headwater streams, since they are usually nutrient limited systems (Burrows et al. 2015). Also, CPOM leachates could be relevant in streams with low capacity to decompose particulate OM inputs, since dissolved C, N, and P from leachates could easily be assimilated by microbial communities (Meyer et al. 1987, Fellman et al. 2009).

Differences of leachates among riparian CPOM types

Our results revealed that the type of CPOM entering into streams was not an important factor influencing the DOC concentrations and quality of the leachates. Similarly, we found that variability in the concentrations of dissolved forms of P among CPOM types was relatively small. In contrast, we found more remarkable differences among CPOM types for dissolved N forms, with higher N concentrations in leachates from alder and black locust leaf litter. This difference could be explained because alder and black locust have the capacity to fix N_2 through their root system (Webster et al. 2009). In this sense, litter inputs from these tree species may alleviate N limitation of heterotrophic microbial assemblages, at least during leaf litter senescence. Overall, these results suggest that the type of riparian CPOM inputs may have stronger relevance for

dissolved N forms than for dissolved C and P forms. Furthermore, we found that leachates from alder had the highest concentrations of NO_3, while NH_4 and DON concentrations were the highest in leachates from black locust. This fact could have an important influence on the balance between NO_3 and NH_4 forms of the recipient streams. In fact, previous studies indicate that NO_3 and NH_4 are cycled differently by streams, because streams have a high capacity to process NH_4 whereas NO_3 tend to be mostly transported to downstream ecosystems (Tank et al. 2008, Ribot et al. 2017). Therefore, the composition and relative abundance of riparian tree species and its associated CPOM inputs may influence not only the DIN pool but also the NO_3:NH_4 ratio, which may have further consequences for in-stream DIN cycling and transport.

Response of microbial activity to different CPOM leachates

In contrast to our expectation, we found a positive relationship between the degree of aromaticity in the CPOM leachates and the microbial activity. This result is also in contrast with studies showing that values of $SUVA_{254}$ are negatively related to the bioavailability of DOM in bulk water samples (Saadi et al. 2006, Fellman et al. 2008), which primarily stems from groundwater and terrestrial runoff. Nevertheless, our results are in agreement with other previous studies assessing the bioavailability of leachates from CPOM (Wymore et al. 2015). This suggests that the relationship between chemical and optical properties of leachates and its bioavailability can vary among DOM sources, which in turn rely on their origin (i.e., as soils or CPOM) (Wymore et al. 2015). We do not have an explicit explanation to this observation, but our results suggest that the variability in heterotrophic microbial activity (inferred from Rru tracer production) measured among CPOM types could be explained by other factors rather than solely DOM aromacity properties. For example, we observed that CPOM leachates

with higher degree of aromacity also contain higher dissolved N concentrations (both as NO_3^- and DON), which explained a remarkable proportion of the variability of Rru production rates among CPOM types. Accordingly, previous studies have shown that respiration rates of stream microbial assemblages are related to N concentrations of CPOM leachates, especially under N-limiting conditions (Kroer 1993, Zweifel et al. 1993). Our results therefore indicate that differences in N concentrations of leachates among CPOM types, especially those related to NO_3^-, could be a relevant factor controlling the bioavailability of these leachate sources to streams, and ultimately, the activity of microbial assemblages of recipient streams. These results complement existing knowledge about the influence of riparian forest composition on the dynamics of stream ecosystems. Previous studies have shown that decomposition of the CPOM in streams vary with the type of CPOM inputs (Webster and Benfield 1986; Cornwell et al. 2008). Here we show that dissolved sources from leachates of different riparian CPOM types can also exert a strong influence on the in-stream microbial activity, especially during periods of leaf litter senescence.

In conclusion, results from this study indicated that riparian input of CPOM to streams is not only a source of particulate matter for in-stream microbial communities, but it can also constitute a relevant source of dissolved solutes such as those from C, N, and P elements. Furthermore, our results suggest that CPOM inputs from riparian vegetation produce leachates of different chemical and structural properties that may influence in-stream microbial activity. Differences in the effect of CPOM inputs among CPOM types are mostly associated to the supply of dissolved forms of N. Due to the rapid nature of the leaching process, the influence of high-quality leachates on microbial communities may be acute but sustained over the leaf senescence period, which can last

over several weeks or months. This reflects the influence of seasonally-driven inputs of CPOM to annual variation of stream dynamics not only related to solutes, but also to microbial activity. Within this context, our results provide support to the relevance of riparian tree composition on the dynamics of stream ecosystems already shown by previous studies. This influence may be especially relevant if the riparian vegetation is dominated by a single species with a narrow phenology in leaf senescence. On the other hand, if the riparian vegetation is composed by a wide range of species with little overlap in leaf senescence, CPOM leachates and their variation in quality and elemental concentrations can be highly important for overall stream microbial activity, especially if several species produce leachates of high quality.

To date, the management of riparian areas has been addressed to provide high-quality litter to streams (i.e., litter with high N content relative to C content and/or low lignin concentrations). Usually, high-quality litter such as alder tends to decompose faster than low-quality litter such as oak or wood (Webster and Benfield 1986; Webster et al. 2009). Thus, the use of certain riparian species can exert strong influences on particulate organic matter dynamics in streams as well as on the composition of microbial and macroinvertebrate decomposers (Webster and Benfield 1986; Webster et al. 2009). Our study also indicates that strategies to manage species composition of vegetation in riparian zones could also have implications for the heterotrophic activity in stream ecosystems associated with dissolved sources of C and nutrients, since changes in riparian vegetation community composition will also likely determine the quantity, quality, and bioavailability of leachates associated with CPOM inputs. Therefore, we recommend considering the composition and relative abundance of riparian tree species since this can have an effect on the properties of the leachates from riparian CPOM inputs, which can affect the heterotrophic activity of recipient streams. Finally, we

suggest that these effects can be especially relevant in streams where the nutrient pool and/or the decomposition of litter inputs are low.

CHAPTER 7: WHEN LEAF LITTER SPECIES MATTER, MICROBIAL UPTAKE OF AMMONIUM AND ACETATE FROM STREAM WATER DURING DECOMPOSITION

7.1. Abstract

The use of isotopically-labeled dissolved forms of carbon (C) and nitrogen (N) has revealed that microbial assemblages developed on decomposing leaf litter in streams can assimilate these elements from the water column. However, most previous studies consider leaf litter as a "black-box" encompassing a mixture of different leaf litter species exposed to different decomposition stages. The present study aims to open this "black-box" to disentangle how the uptake of $N\text{-}NH_4$ and acetate from water column vary among riparian leaf litter species differing in the initial quality (i.e., C:N ratio) and under different stages of decomposition. We exposed leaf litter of 5 riparian tree species to different times of incubation in a stream and conducted a 24h addition of $^{15}N\text{-}NH_4$ and ^{13}C-acetate at constant rate to estimate the uptake of these solutes. In addition, we measured the microbial exoenzymatic activity of cellobiohydrolase (CBH) of microbial assemblages on leaf litter to examine its influence on the uptake of $^{15}N\text{-}NH_4$ and ^{13}C-acetate. We found that N uptake was relatively similar over leaf litter decomposition period, whereas C uptake increased. These patterns were similar among leaf litter species, but rates significantly differed among them. In addition, the average uptake of NH_4 for each leaf litter species was negatively related with the decomposition rate and the accumulated CBH activity, whereas the uptake of acetate was positively related to these variables. This suggests that the type of leaf litter inputs can be important to determine the interaction between microbial assemblages on leaf litter and water column chemistry, and that this influence may depend on the element considered. Ultimately, our results show that the leaf litter inputs from the riparian forest are not only relevant as a source of particulate organic matter to streams contributing to stream metabolism, but that the species composition of riparian forest can also influence the

cycling and downstream export of dissolved inorganic N and organic C of recipient stream ecosystems.

7.2. Microbial uptake of N and C from the water column

Microbial assemblages in streams can use leaf litter as a substratum for colonization as well as a source of carbon (C) and nutrients for metabolic activity and growth; and thus, they can contribute to the decomposition of leaf litter inputs from riparian zones. In this sense, riparian leaf litter inputs have been shown to fuel the ecosystem metabolism especially of headwater-forested streams (Fisher and Likens 1973, Vannote et al. 1980). Microbial assemblages developed on leaf litter (mainly fungi and bacteria) produce specific extracellular enzymes that breakdown C-polymeric compounds form leaf litter into smaller molecules that can be more easily assimilated (Chróst 1991; Romaní et al. 2006; Romaní et al. 2012). Bacteria developed on leaf litter can also take up dissolved organic carbon (DOC) from the water column during the decomposition process (Hall and Meyer 1998; Pastor et al. 2014). In addition, nutrients (mainly nitrogen [N] and phosphorous [P]) provided by leaf litter may not fulfill microbial demands; and thus, microbial decomposers often need to acquire nutrients from the water column (Kaushik and Hynes 1971; Webster and Benfield 1986; Sampaio et al. 2001; Gulis and Suberkropp 2003). Therefore, during the leaf litter decomposition process, microbial demand of C and nutrients can be supplied from either leaf litter or from the water column and this supply may change over time depending on the characteristics of the leaf litter substrate (Webster et al. 2009).

The uptake of C and nutrients from water column by in-stream biotic primary uptake compartments has been mostly examined by using additions of isotopically labelled C and N dissolved forms (Hall and Meyer 1998, Tank et al. 2018), which allow tracing the transfer of elements from water column into uptake compartments without modifying ambient concentrations. Most of these studies have focused on N uptake, and have showed that the relative contribution of leaf litter compartment to the total in-stream N uptake is highly variable among streams (i.e., from 0 to 60%). This suggests that particular environmental conditions of streams and/or the intrinsic properties of leaf litter may influence N demands of microbial assemblages on leaf litter from the water column. These studies usually consider in-stream leaf litter as a "black-box" primary uptake compartment, although this compartment is often constitute by a mixture of different leaf litter species which can also be exposed to different stages of decomposition. In this regard, Webster et al. (2009) suggested that the relative dependence of dissolved nutrients from either leaf litter and water column by microbial decomposers can be determined, at least in part, by the leaf litter quality. In addition, results on how the dependence of microbial decomposers on N and C from water column varies over the decomposition period are controversial. Some studies indicate that N uptake is high during the initial stages of decomposition (Tank et al. 2000; Valett et al. 2008) and that it decreases over leaf litter decomposition as microbes can access nutrients from leaf litter (Webster et al. 2009). In contrast, Cheever et al. (2013) showed that microbial assemblages on decomposing leaves acquired more N from water column as decomposition stages advance. In this study we examine how that variation in the quality of leaf litter either associated with different tree species or with different decomposition stages can explain contrasted results on the interaction between leaf litter decomposition and C and nutrient uptake from water column.

Quality of leaf litter can be determined by its elemental composition (i.e., the content of C, N and P) and the relative proportion among these elements (Melillo et al. 2001). Leaf litter with high N and P content relative to C content commonly decomposes faster than leaf litter with low relative content of N and P (Webster and Benfield 1986; Enriquez et al. 1993). Other indicators of leaf litter quality are related to the complexity of leaf litter organic C molecules (Webster and Benfield 1986). In this sense, soluble polysaccharides are simple organic compounds, which are easily degraded and consumed by microbes; and thus, they are considered as labile C sources. In contrast, lignin or tannins are metabolically more costly to be used by microbes; and thus, considered as recalcitrant C resources (Sinsabaugh et al. 1993). In this regard, leaf litter quality has been shown as a relevant factor controlling the dynamics of microbial colonization and metabolic activity associated with decomposing leaf litter. Previous studies indicated different microbial colonization patterns between leaf litter species differing in their nutrient content (Webster et al. 2009) or in their content of recalcitrant compounds (Gessner and Chauvet 1994) because these factors can limit growth and activity of fungi on leaf litter (Canhoto and Graça 1999) and microbes.

The main mechanisms by which microbial decomposers degrade leaf litter polymeric compounds is the production of exoenzymes (Rogers 1961; Artigas et al. 2008). The amount of exoenzymatic activity produced by microbial decomposers can widely vary among leaf litter species, which suggests that leaf litter quality can be a primary mechanism by which microbial decomposers degrades leaf litter polymeric compounds (Sinsabaugh et al. 1994; Romaní et al. 2004). Additionally, it has been observed that microbial benthic communities colonizing organic substrates are partially reliant on organic material and inorganic nutrients supplied by the surrounding water (Sala et al.;

Romaní et al. 2004, 2012). Therefore, the production of microbial enzymatic activities can be modulated by both C and nutrients from leaf litter and from water column (Romaní et al. 2014).

The present study aims to understand how the uptake of DIN (i.e., N-NH_4) and DOC (i.e., acetate) from water column vary among riparian leaf litter species, which differ in the initial quality (i.e., C:N ratio), and among different stages of leaf litter decomposition. Additionally, we explored whether variation in the uptake of N-NH_4 and acetate among leaf litter species are related to the activity of the microbial decomposers developed on leaf litter as expressed by the exoenzymatic activity of cellobiohydrolase (CBH). We expected that: (i) microbial decomposers developing on low-quality leaf litter (i.e., high C:N ratio) would have higher N-NH_4 and C-acetate uptake rates than those developing on a high-quality leaf litter and that (ii) microbial decomposers on leaf litter would increase their demand of N-NH_4 and C-acetate from the water column at later stages of the decomposition process because C and N contained in leaf litter are used over the decomposition process.

7. 3. Methods

Description of the experimental design

The study was conducted in an irrigation channel (200 m long and 2 m wide), which receives water from the Font del Regàs stream, a 3rd order tributary of La Tordera river (NE Spain; 41º50´N, 2º30´E, 300 m a.s.l.). The water input to the channel was regulated by a floodgate, which allowed keeping the water discharge in the channel constant (~25 L s^{-1}) during the entire study period (from 28th Nov 2013 to 25th Jan 2014). The channel

morphology is characterized by small riffles and pools and a bed substratum composed by sand and cobbles.

We used stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) as tracers to quantify the uptake of N and C from water column into the microbial assemblages developed on leaf litter during the decomposition period. In particular, we measured microbial uptake of $^{15}N\text{-}NH_4$ and ^{13}C-acetate using a constant rate addition of N and C stable isotopes following methods by Tank et al (2017) and Hall and Meyer (1998). Microbial uptake of $^{15}N\text{-}NH_4$ and ^{13}C-acetate was measured in leaf litter from 5 different species of riparian trees and at several stages of the leaf litter decomposition. Riparian tree species considered in this study were: alder (AL, *Alnus glutinosa*); ash (AS, *Fraxinus excelsior*); black poplar (BP, *Populus nigra*); black locust (BL, *Robinia pseudoacacia*); and sycamore (SY, *Platanus x hispanica*). These species are representative of riparian zones from headwater streams of the Mediterranean temperate region, where the study was conducted.

For this study, leaf litter from the different riparian tree species was collected during the leaf fall period (i.e., November 2013) using traps placed over the Font del Regàs stream. Leaves were transported to the lab, air dried at lab conditions (25 ºC and 30% humidity), and stored. To expose leaf litter to in-stream microbial decomposition, we placed ~4.5 g of air-dried leaves in 250 µm mesh-size bags to ensure minimal influence of macroinvertebrates during decomposition process. Leaf bags (n=20, 5 species x 4 replicates per incubation time) were placed along the study channel at 75, 45, 30, 10 and 2 days prior the addition of N and C stable isotopes. Therefore, by the time of the stable isotope addition in the stream there were samples of decomposing leaf litter at 5 different decomposition stages for the 5 different species. To avoid the influence of physical abrasion during leaf litter decomposition, leaf bags were placed in sampling

sites of relatively slow water velocity (from 5 to 10 cm s^{-1}). During the study period, water temperature and water depth were recorded every 20 minutes using waterproof temperature data loggers (HOBO Pendant® UA-002-64) and a pressure data logger (Solinst Levelogger Junior Edge), respectively. We also measured water velocity and dissolved oxygen concentration every 10 days at the location of each leaf bag using a velocity-meter (Miniair20/Schiltknecht) and WTW (Weilheim, Germany) 340i portable sensor, respectively. On each date when leaf bags were placed in the channel, we estimated discharge using a mass balance approach by adding 1 L of a NaCl-enriched solution to the channel (Gordon et al. 2004). On these dates, we also collected water samples at 3 equidistant points along the 200-m channel to analyze concentrations of dissolved organic C (DOC), nitrate (NO_2 + NO_3), ammonium (NH_4), and phosphorous (as soluble reactive phosphorous; SRP). All water samples were immediately filtered through ashed (500 ºC during 5 hours) FVF glass filters (0.7 µm pore size) and kept on ice until arrival to the lab, and then stored at -20ºC until analysis (see below).

In-stream addition of ^{15}N-NH_4 and ^{13}C-acetate

Two days after the last placement of leaf bags into the channel, we conducted a ~24h constant rate addition of ^{15}N-NH_4 and ^{13}C-acetate (as 99% enriched $^{15}NH_4Cl$ and $^{13}CH_3COONa$, respectively). On the addition date, the study reach contained leaf bags of the 5 different leaf litter species exposed to 5 different dates over the decomposition process. The ^{15}N-NH_4 and ^{13}C-acetate addition was designed to increase the isotopic signatures of ^{15}N and ^{13}C by 1000 fold and 100 fold, respectively, while keeping ambient N-NH_4 and DOC concentrations in the water. We assumed uptake of ^{13}C-acetate was mostly associated with bacteria because the concentration of acetate during the addition (~0.09 µmol L^{-1}) was higher than that needed to be assimilated by bacteria

(0.001 µmol L^{-1}; Newell 1984), but lower than that needed to be assimilated by fungi (0.1-1 mmol L^{-1}; Wright and Hobbie 1966; Hall and Meyer 1998). Water was collected prior to the stable isotope addition (i.e., background sampling) and ~24h after the beginning of the addition (i.e., plateau sampling) at 6 different stations along the channel to determine the signature of ^{15}N-NH_4 and ^{13}C-acetate under background and plateau conditions, respectively. We collected 4L and 1L of water per station for analysis of ^{15}N and ^{13}C, respectively (only one replicate per station). At each station, we also collected water (15ml, two replicates per sampling station) to determine nutrient and DOC concentrations. All water samples were immediately filtered through ashed (500 ºC during 5 hours) FVF glass filters (0.7 µm pore size). Samples for ^{13}C were acidified with 10% HCl to remove dissolved inorganic C. Filtered samples for nutrient chemistry and ^{13}C were kept on ice until arrival to the lab, and then stored at -20ºC until analysis. Samples for ^{15}N-NH_4 were immediately processed (see procedure below).

On the background and plateau samplings we also collected leaf bags (1 replicate per leaf litter species and incubation time at background and 3 replicates at plateau) to estimate the signatures of ^{15}N and ^{13}C and CBH enzyme activity in leaf litter. Leaf bags were carefully rinsed with water from the channel (upstream of the addition point) to remove sediment attached to the bag surface. Then, leaf litter was removed from the bags and a sub-sample was collected for the analysis of isotopic N and C signatures. In addition, a 14 mm diameter disc of leaf litter for all the species and incubation times was sampled for posterior analysis of CBH enzyme activity. All leaf litter samples were stored at ~4ºC until further analysis.

Laboratory analyses

Water samples were analyzed for NO_3, NH_4, and SRP following standard colorimetric methods (APHA 1995) on an Automatic Continuous Flow FUTURA–ALLIANCE Analyzer at the CEAB-CSIC (Blanes, Spain). Concentration of DOC was determined by Shimadzu TOC-V CSH analyzer (Shimadzu Corporation, Kyoto, Japan) at the Serveis Cientificotècnics of the University of Barcelona (Barcelona, Spain). The ^{15}N-NH_4 signature of water samples was determined following the ammonia diffusing procedure adapted from Holmes et al (1998). Briefly, we added 3 g L^{-1} of MgO and 50 g L^{-1} of NaCl to water samples and a Teflon filter packet containing a 1-cm-diameter combusted Whatman GF/D fiber glass filter acidified with 25 µL of 2.5 M $KHSO_4$ (to trap the volatilized NH_3). Water samples were incubated on a shaker at 40ºC for 4 wk. After the incubation, we removed the filter packets and placed them in a desiccator for 5 d. We encapsulated filters in tins and analyzed the ^{15}N:^{14}N ratio by Elemental Analysis - Isotope Ratio Mass Spectrometry (EA-IRMS) at the Serveis Cientificotècnics of the University of Barcelona. For ^{13}C water analysis, water samples were bubbled for 6 minutes with compressed nitrogen gas (N_2) to remove gaseous inorganic C retained after acidification. Then, the ^{13}C:^{12}C ratio was analyzed by Flow Injection Analysis–IRMS.

Leaf litter samples were oven dried at 60 ºC until constant weight and weighted to estimate remaining dry mass (DM). After weighted, leaf litter was crushed into a fine powder using a grinder mill (Biometa MM 200). Subsamples of ~1.5 mg were weighted to the nearest 0.001 mg with MX5 microbalance (Mettler-Toledo, Greinfense, Switzerland), encapsulated into tin capsules, and sent to the Serveis Cientificotècnics of the University of Barcelona to determine both, N and C content (mg N g DM^{-1} and mg C g DM^{-1}, respectively), and ^{15}N and ^{13}C stable isotope signatures (same techniques as

for filter packets). Stable isotope signatures of ^{15}N and ^{13}C are expressed as δ values in per mil units (‰) using international reference stable isotope standards of air for N and Vienna Pee Dee Belemnite for C.

The CBH extracellular enzyme activity was quantified using the methylumbelliferyl (MUF) fluorescent-linked substrate method (Romaní et al. 2006). The assays were conducted at MUF saturation conditions of 0.5 mM. Briefly, leaf litter discs and water controls were incubated with MUF-linked substrate for 1 hour in a dark in a shaker. Blanks and standards of MUF (0–100 μmol L^{-1}) were also incubated. At the end of the incubation, Glycine buffer (pH 10.4) was added (1/1 vol glycine/vol sample), and the fluorescence was measured at 365/455 nm excitation/emission (Kontron SFM25 fluorimeter). The CBH extracellular enzyme activity was expressed as the amount of MUF substrate produced per incubation time and leaf litter dry mass (DM) (in μmols MUF h^{-1} g DM^{-1}). Higher values of MUF produced indicate higher production of CBH by microbes; and thus, higher extracellular activity.

Data analysis

For each leaf litter species, the decomposition rate (*k;* in d^{-1}) was estimated by fitting the remaining DM at each incubation time to a negative exponential model (1) as described in Petersen and Cummins (1974):

$$W_t = W_0 * e^{-k\,t} \qquad (1)$$

where W_0 and W_t are leaf litter DM (in g) at the beginning and at each incubation time, respectively, and t is the incubation time (in d). Values of *k* denote the velocity at which leaf litter mass decreases over time.

We estimated the accumulated enzyme activity of CBH (AEA, in mmol of MUF g DM^{-1}) over the leaf litter decomposition period by linearly integrating the instantaneous CBH enzyme activity between consecutive incubation time intervals (Simon and Benfield 2009). We also calculated the CBH use efficiency as the turnover activity (TA, in mmol MUF g DM^{-1}), which is the amount of CBH produced to decompose 1 g of leaf litter (Simon and Benfield 2009). TA was calculated as the inverse of the slope of the regression between the amount of remaining leaf litter (y) and the accumulated enzyme activity (AEA) for each incubation time (x). High values of TA denote that the enzyme produced is not efficient to decompose leaf litter, while low values indicate the opposite.

The uptake rates for a given isotopically labelled element (i.e., ^{15}N, ^{13}C) are usually reported as a nutrient mass per unit of area and time (Mullholand et al. 2000; Peipoch et al. 2016). However, during our ^{15}N and ^{13}C addition, leaf litter samples contained different DM and N and C content depending of both, leaf litter species and incubation times. Therefore, we expressed the uptake of ^{15}N and ^{13}C of each leaf litter sample in terms of biomass-specific uptake (i.e., U-NH_4 and U-acetate, respectively) so results could be compared among leaf litter species and decomposition stages. We calculated the biomass-specific N uptake (in µg N mg N^{-1} d^{-1}) of leaf litter at each incubation time for each species following the equation:

$$Biomass - specific\ \ N\ uptake\ rate =$$

$$[((\delta\ ^{15}N/1000) \times 0.003663\ \times N_{biomass})/(((\delta\ ^{15}N - NH_4/1000)\ \times\ 0.003663) \times T)]/\ N_{biomass} \quad (3)$$

where $\delta^{15}N$ is the ^{15}N enrichment of leaf litter at the plateau conditions (in ‰), $N_{biomass}$ is the standing stock of N in leaf litter (in µg N), $\delta^{15}N$-NH_4 is the background-corrected

$\delta^{15}N$ of water at plateau conditions, and T is the elapsed time from the start of the addition to the leaf litter collection (~24 hours).

Similarly, we calculated biomass-specific C uptake (in mg C g C^{-1} d^{-1}) for each leaf litter species at each incubation time following the equation:

$$Biomass - specific\ C\ uptake\ rate =$$

$$[(((\delta\ ^{13}C/1000) \times 0.011237\ \times C_{biomass})/(((\delta\ ^{13}acetate/1000)\ \times 0.011237\ \times\ T)]\ /C_{biomass} \quad (2)$$

where $\delta^{13}C$ is the ^{13}C enrichment of leaf litter at the plateau conditions (in ‰), $C_{biomass}$ is the C standing stock in leaf litter (in mg C), $\delta^{13}C$-acetate is the background-corrected $\delta^{13}C$ of water at plateau conditions and, T is the elapsed time from the start of the addition to the leaf litter collection (~24 hours).

For each leaf litter species, we calculated the integrated values of biomass-specific N uptake of ^{15}N-NH_4 (µg N mg N^{-1}) and of ^{13}C-acetate (mg C g C^{-1}) for the entire study period by linearly integrating the instantaneous U-NH_4 and U-acetate over the incubation period.

Statistical analysis

One-way ANOVA was used to test differences in initial leaf litter C and N content and C:N ratio among the 5 leaf litter species studied. The ANOVA model includes these parameters as dependent variables and leaf litter species (n=5) as fixed factor. Tukey's Honestly Significant Difference pairwise comparisons were then used to determine specific differences in these parameters among leaf litter species.

We used a one-way ANCOVA analysis to explore differences in *k* between the 5 leaf litter species. Remaining mass of each leaf litter species was log-transformed prior to the analysis. The one-way ANCOVA included remaining mass as dependent variable and incubation time (expressed in days) as the covariate variable. Leaf species (n=5) was the fixed factor. The interaction term (leaf litter species x incubation time) was used to explore whether the loss of leaf litter mass over incubation time was similar among leaf litter species (Zar 1999). We used Tukey´s post hoc-test to determine specific statistical differences in *k* among leaf litter species.

We used two-way ANOVA analyses to explore differences in leaf litter C and N content, CBH exoenzymatic activity, biomass-specific N-NH_4 and C-acetate uptake among species and over the incubation time. These variables were included in the model as dependent variables while leaf litter species and incubation times were included as fixed factors. For each variable, the interaction term (incubation time x leaf litter species) was used to determine whether differences among incubation times were consistent among leaf litter species.

We used linear models to explore whether variation in the integrated values of biomass-specific N and C uptake over the decomposition period were related to quality and functional characteristics of leaf litter such as initial C:N ratio of leaf litter, leaf litter *k*, accumulation of CBH exoezymatic activity (AEA) and turnover enzyme activity, as a surrogate of CBH use efficiency.

7.4. Results

Environmental characterization during leaf litter decomposition period

During this study, leaf litter in the stream was exposed to a relatively low discharge and water temperature (Table 7.1.). Concentrations of NO_3, NH_4 and SRP in stream water were low and remained relatively constant over the decomposition period (Table 7.1.). The concentration of NO_3 accounted for the largest fraction of the dissolved inorganic N concentration (i.e., NO_3 + NH_4; Table 7.1.). All in-stream sites where leaf bags were deployed were well oxygenated and exposed to relatively low water velocity (Table 7.1.).

Table 7.1. Mean values for physical and chemical parameters of stream during the leaf litter incubation period. The SE of the mean is shown in parenthesis. * indicates measurements done at each leaf litter deploying sites.

Parameter	Mean (SE)
Discharge (L s^{-1})	25 (2.2)
*Water velocity (cm s^{-1})	2.8 (0.22)
Temperature (ºC)	5.4 (1.3)
N-NH_4^+ (µg N L^{-1})	2.6 (1.8)
N-NO_3^- (µg N L^{-1})	194.2 (87)
SRP (µg P L^{-1})	212.6 (15)
*DO (mg L^{-1})	11 (0.1)
*DO (%)	93.5 (1.7)

Characterization of leaf litter species

The initial leaf litter C and N content differed among the leaf litter species (one-way ANOVA, $p = 0.008$ for C and $p < 0.001$ for N). Dry leaves from AS, AL and BL showed the highest content of N and the lowest C:N ratio (Table 7.2.). In contrast, leaves from SY showed the highest C content and C:N ratio (Table 7.2.). During decomposition process, the C content of leaf litter was similar among leaf litter species (two-way ANOVA, $p > 0.05$) and remained relatively constant over incubation time (two-way ANOVA, $p > 0.05$). In contrast, the N content increased over the incubation time (two-way ANOVA, $p < 0.001$). This trend was common among leaf litter species, except for BL for which N content remained relatively constant over time. In addition, N content varied among leaf litter species (two-way ANOVA, $p < 0.001$) with leaves from AL showing the highest values over the entire study period followed by leaves from AS, BL and BP. Leaves from SY had the lowest N content during the study period.

The CBH enzyme activity increased over the leaf litter decomposition period regardless of the leaf litter species considered (two-way ANOVA, $p < 0.001$). In addition, CBH enzyme activity differed among leaf litter species (two-way ANOVA, $p < 0.001$), being lower for SY and higher for BL (Table 7.2.). In this sense, the AEA of CBH over the incubation period ranged from 5.5 to 1.3 mmols MUF g DM^{-1} for the species considered (Table 7.2.). The highest and lowest values of TA of CBH were observed in BL and AS, respectively (Table 7.2.).

Table 7.2. Mean values of the initial C:N molar ratio, cellobiohydrolase (CBH) enzyme activity, accumulated enzyme activity for CBH, CBH enzyme use efficiency calculated as turnover activities (TA) and leaf litter decomposition rates (*k)* for the 5 leaf litter species considered in the study. The SE of the regression (for *k*) and of the mean (for the rest of variables) are shown in parenthesis. For each variable, different letters indicate statistical significant differences among tree species based on Tukey´s post hoc-tests after applying one-way ANCOVA test (for *k*) and one-way ANOVA test (for the rest of variables).

Species	C:N ratio	CBH activity (μmols MUF g DM^{-1} h^{-1})	AEA (mmols MUF g DM^{-1})	TA (mmols MUF g DM^{-1})	*K* (day^{-1})
Ash (AS)	17.3 (0.3)^{a}	1.3 (0.3)^{bc}	3.6	2	0.0163 (0.0021)^{a}
Alder (AL)	16.8 (1.1)^{a}	1.4 (0.2)^{bc}	2.8	2.3	0.0103 (0.0014)^{b}
Black poplar (BP)	26.5 (1.5)^{b}	1.6 (.0.4)^{ab}	2.9	4	0.0080 (0.0006)^{b}
Black Locust (BL)	17.4 (0.9)^{a}	2.4 (0.4)^{a}	5.5	12.3	0.0054 (0.0007)^{c}
Sycamore (SY)	46.8 (0.3)^{c}	0.8 (0.1)^{c}	1.3	7.9	0.0019 (0.0002)^{d}

The leaf litter mass of the 5 studied species placed in the stream decreased over incubation time following an exponential decay model (in the 5 cases $0.76 < r^2 < 0.82$; $p < 0.001$). Values of *k* differed among leaf litter species, ranging from 0.0163 day^{-1} (AS) to 0.0019 day^{-1} (SY) (Table 7.2.). Leaf litter *k* was positively related with AEA ($r^2 = 0.85$, $p < 0.05$; data not shown), though the relationship was not significant when including data from BL leaf litter in the analysis. In addition, there was a negative, though no statistically significant, relationship between *k* and TA ($p = 0.08$). Values of *k* for the different leaf litter species were not statistically related with their initial N content nor with the C:N ratio.

Uptake of NH_4 and acetate from water column during leaf litter decomposition

The biomass-specific uptake of NH_4 (U-NH_4) was relatively stable during the leaf litter decomposition period (Figure 7.1.; left panels). Values of U-NH_4 were remarkable even at early decomposition stages, and differed among incubation times (two-way ANOVA, $p < 0.001$) but did not show any clear trend. The highest and lowest values were measured at day 45 and 30/75, respectively (Figure 7.2. A). In addition, U-NH_4 was highest for leaf litter from SY and lowest for leaf litter from AS, AL and BP (two-way ANOVA, $p < 0.001$; Figure 7.2. C). The interaction term (incubation time x leaf litter species) was not significant, indicating that temporal variability of U-NH_4 during decomposition was similar among leaf litter species (Figure 7.1., left panels).

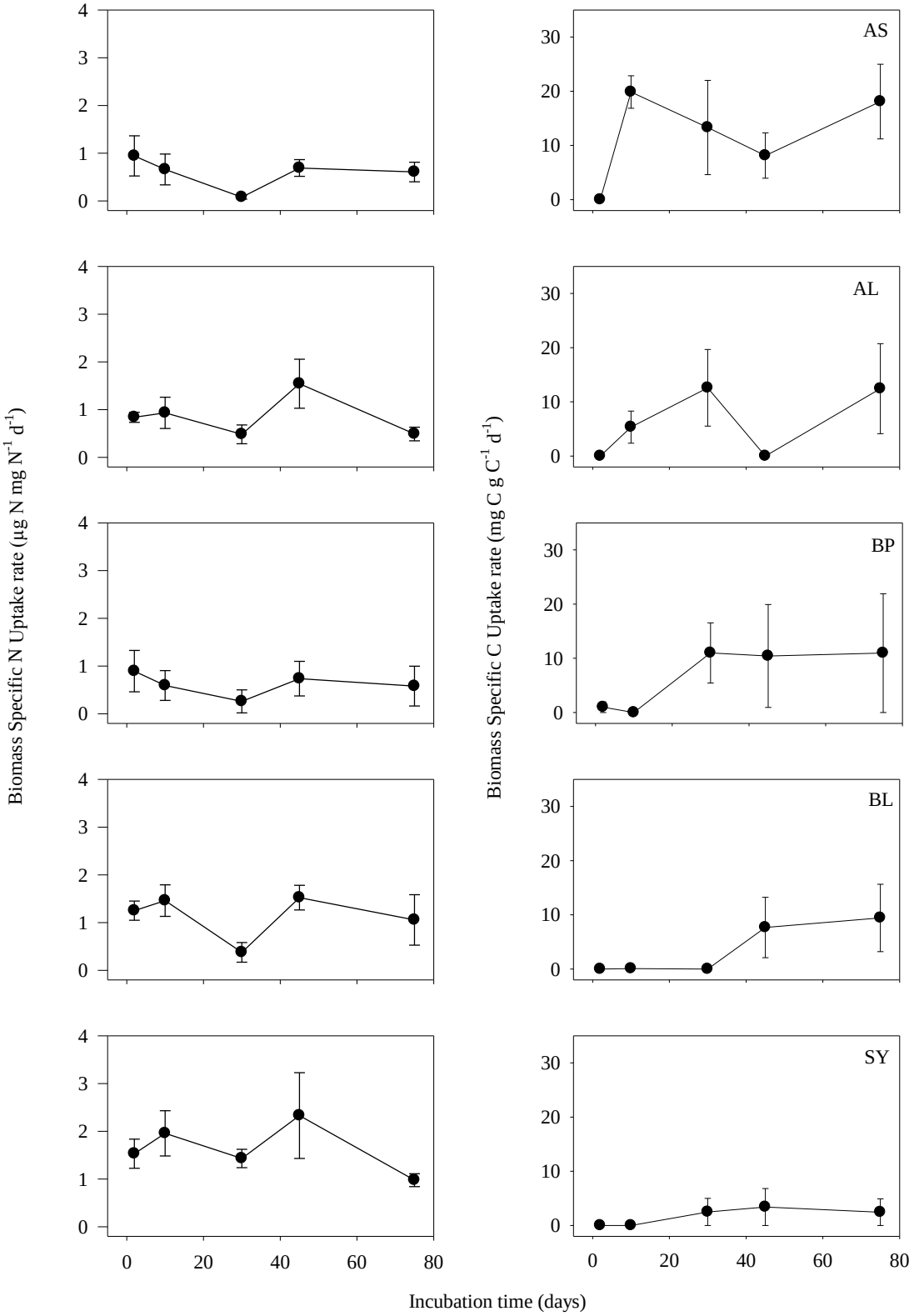

Figure 7.1. Temporal variation of biomass specific NH_4 uptake rates (left panels) and biomass specific acetate uptake rates (right panels) during decomposition for 5 leaf litter species (n=3 for each incubation time). AS (ash), AL (alder), BP (black poplar), BL (black locust) and SY (sycamore). Data points are means and vertical bars represent SEs.

In contrast, biomass-specific uptake of acetate (U-acetate) increased during leaf litter decomposition (two-way ANOVA, $p < 0.05$) being highest after 75 days of incubation (Figure 7.2. B panels) regardless of leaf litter species considered. Values of U-acetate also differed among leaf litter species, being highest for leaf litter from AS and the lowest for leaf litter from BL and SY (two-way ANOVA, $p < 0.05$; Figure 7.2. D). The interaction term (incubation time x leaf litter species) was not significant, indicating consistent temporal patterns over decomposition process of U-acetate among different leaf litter species (Figure 7.1., right panels).

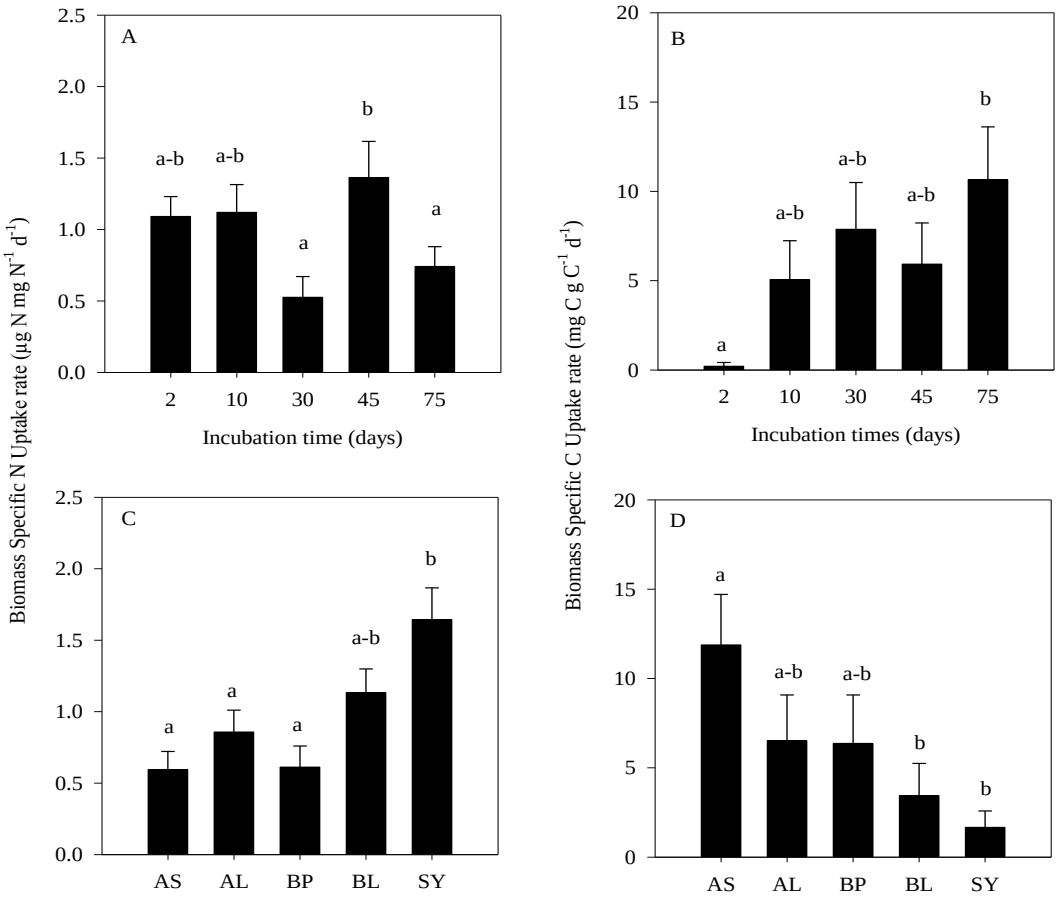

Figure 7.2. Mean and SE of biomass specific NH_4 uptake rates (A) and biomass specific acetate uptake rates (B) for each incubation time and of biomass specific NH_4 uptake rates (C) and biomass specific acetate uptake rates (D) for each leaf litter species. Lowercase letters indicate statistical differences on the uptake rates among incubation times and leaf litter species base on two-way ANOVA models followed by Tukey´s post hoc-test. Incubation time and leaf litter species were considered as factors in the model. AS (ash), AL (alder), BP (black poplar), BL (black locust) and SY (sycamore).

We found that integrated values of U-NH_4 and U-acetate over the decomposition period for each leaf litter species were negatively related (Pearson correlation, r = 0.79, p = 0.02). In addition, integrated U-NH_4 and U-acetate were not related to the initial leaf litter C:N ratio (Figure 7.3. A-B). However, integrated U-NH_4 was negatively related to k (r^2 = 0.8, p = 0.03; Figure 7.3. C) and AEA (r^2 = 0.98, p = 0.006; Figure 7.3. E), and positively related to TA (r^2 = 0.87, p = 0.04; Figure 7.3. G), only when values from BL were excluded. In contrast, integrated U-acetate was positively related to k (r^2 = 0.91, p = 0.008; Figure 7.3. D) and AEA (r^2 = 0.96, p = 0.02; Figure 7.3. F), only when values from BL were excluded.

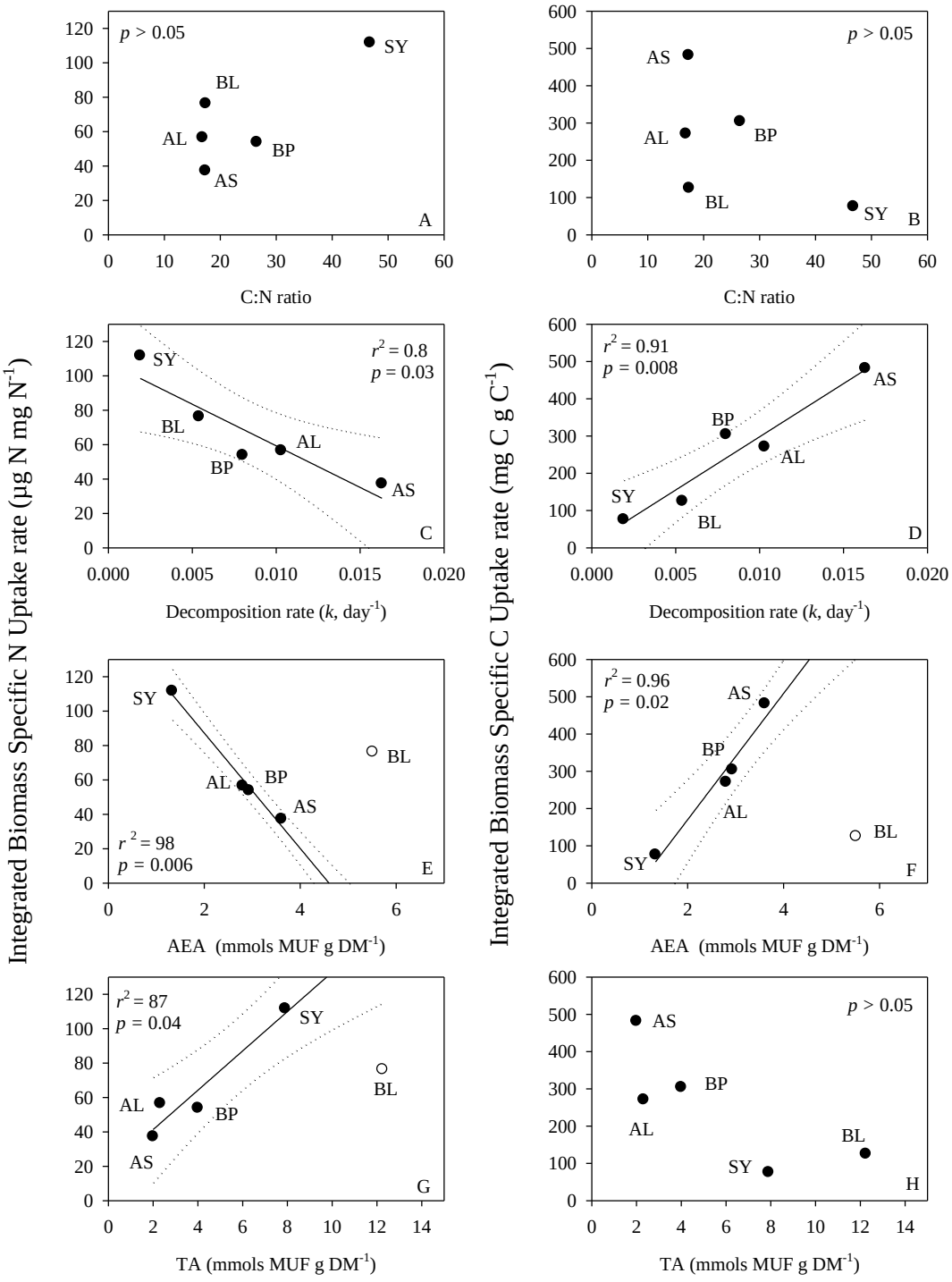

Figure 7.3. Linear relationships between the integrated values of both biomass specific NH_4 uptake rates and biomass specific acetate uptake rates during the incubation time (i.e., Integrated Biomass Specific N Uptake rates and Integrated Biomass Specific C Uptake rates, respectively) with initial values of the C:N ratio of leaf litter (A-B), leaf litter decomposition rates (*k*) (C-D), accumulated enzyme activity (AEA) of cellobiohydrolase (CBH) (E-F) and CBH enzyme use efficiency calculated as turnover activity (TA) (H-I). The coefficient of determination of the regressions (r^2) and the p-value (*p*) are shown. Values of Black locust (BL) leaf litter species were indicated in bold when linear significance between variables was achieved excluding BL species. AS (ash), AL (alder), BP (black poplar) and SY (sycamore).

7.5. Discussion

The influence of dissolved nutrients and carbon from water column on leaf litter decomposition is a relevant topic that has been widely assessed in stream ecosystems (Suberkroop and Chauvet, 1995; Woodward et al. 2012; Ferreira et al. 2014; Bastias et al. 2017). Yet, studies particularly quantifying solute fluxes from the water column to microbial decomposers are less common, despite these studies suggest that microbes on leaf litter can contribute to DIN retention from the water column (Dodds et al. 2000; Tank et al. 2000). In fact, a recent study considering a wide variety of headwater streams shows that decomposing leaf litter can account on average for ca. 15% of the total ^{15}N added that is stored in a stream reach (Tank et al. 2017). These studies have commonly considered the leaf litter compartment as a "black box", which includes a mixture of different leaf litter species exposed to different decomposition stages. Results of our study open this "black box" and provide information on how different leaf litter species contribute to the uptake of NH_4 and acetate from the water column, and how this contribution can vary over different decomposition stages. The use of ^{15}N and ^{13}C stable isotopes revealed that NH_4 and acetate uptake associated with decomposing leaf litter differed among species. In addition, species with higher uptake of NH_4 showed lower uptake of acetate and viceversa. Uptake of NH_4 and acetate also varied during the leaf litter decomposition process, especially for acetate, which tends to increase under advanced stages of decomposition.

A fraction of the ^{15}N-NH_4 added to the water during 24 h was detected in the leaf litter of the 5 studied species even at the very early stages of leaf litter decomposition (i.e., two days of incubation). These results reinforce the notion that microbial assemblages

developed on leaf litter can rely on DIN from the water column to satisfy their N demand (Mulholand et al. 1985; Webster et al. 2001; Mulholand 2004). These results also indicated that microbial decomposers use N from water column even at early colonization stages regardless of leaf litter species, as suggested in previous studies (Tank et al. 2000; Webster et al. 2009). In addition, the uptake of NH_4 showed small variation over the decomposition period indicating that N demands from the water column are also needed as leaf litter decomposition proceeds. This result contrasts with the assumption that assimilation of NH_4 is mostly associated with fungi colonizing leaf litter during initial decomposition stages (Suberkrop and Klug 1976; Webster et al. 2009), and suggests that bacteria, which colonize leaf litter at later stages, can also uptake DIN from the water column. The increase in acetate uptake during the decomposition process additionally suggests that bacteria on leaf litter also rely on C from the water column. In addition, the concentration of acetate used was too low to be assimilated by fungi; and thus, bacterial uptake should be the major contributor of C uptake from stream water measured over the decomposition process, as suggested by previous studies (Wright and Hobbie 1966; Hall and Meyer 1998). A higher demand of C-labile compounds, such as acetate, from water column could also be explained by the decrease in C-labile resources from leaf litter tissues as decomposition advances.

As expected, our results indicated that C and nutrient uptake associated with leaf litter not only depend on the decomposition stage, but also on the quality of the leaf litter where they develop. However, NH_4 and acetate uptake were oppositely related among leaf litter species, which contrast with our expectations. In addition, the initial C:N ratio of leaf litter was not a significant predictor of the C and N demands from water column of the different species. This may be explained because the quality of leaf litter can be determined by other factors beyond the C:N ratio such as the type of C molecules

constituting the leaf litter or by a combination of these factors. For instance, leaves of sycamore contain a high proportion of C-recalcitrant compounds (Gessner and Chauvet 1994), which could explain the high dependence of N from water column as well as the low leaf litter *k*. In fact, the uptake of NH_4 was negatively related to leaf litter *k* indicating that microbes colonizing poor-quality leaf litter depend in a greater extent on DIN from water column than microbes colonizing high-quality leaf litter. The low NH_4 uptake associated with high-quality leaf litter, such as that from alder, agrees with previous findings from Webster et al. (2009). These authors suggested that nutrients are initially taken from the leaf litter and nutrient uptake from the water column only occurs if needed. This is further supported by the positive relationship between NH_4 uptake and the CBH use efficiency (i.e., TA) we found, which suggests that when microbial decomposers can efficiently use nutrients from leaf litter tissues they rely less on the NH_4 from the water column. In contrast, we found that uptake of acetate was positively related to leaf litter *k* and to the integrated enzyme activity of CBH over the decomposition period (i.e., AEA), which indicates that microbial decomposers acquire more acetate from the water column when they efficiently consume the leaf litter substratum. This finding could be explained by the rapid consumption of C-labile resources on these species, which leads to high *k*. Thus, since acetate is a labile C resource in the water column its uptake by microbial decomposers on leaf litter can contribute to their activity over the leaf litter decomposition process. A remarkable exception of general observed trends by different leaf litter species is results associated with leaf litter from black locust. Decomposition of this leaf litter species showed high values of cumulative exoenzyme production of CBH and NH_4 uptake, which coincide with low acetate uptake and *k*. This suggests that the enzymatic activity in black locust may be sustained by organic compounds other than those from leaf litter (i.e., from

water column), but not by acetate. Moreover, the N demand associated with this activity could be partially linked to NH_4 uptake from water column. A possible explanation to this result could be that black locust can inhibit microbial activity due to the presence of polyphenols (Alonso et al., 2010), which forms complexes with proteins that are highly resistant to microbial activity and decomposition (Taylor et al. 1989; Hattenschwiler and Vitousek 2000). In this sense, microbial decomposers could use black locust mostly as a substrate, but supporting their metabolic activity by solutes from the water column.

In conclusion, our results remark the relevance of leaf litter quality, regardless of the stage of decomposition, on demand of C and N from the water column by microbial leaf litter decomposers. Therefore, in forested headwater streams the composition of the riparian forest can exert strong influences on in-stream DIN and DOC cycling, because it determines the quality of leaf litter inputs not only based on C:N ratios, but also on the C molecular composition of leaf litter tissues. Furthermore, we found that leaf litter quality may influence C and N cycling in streams in opposite ways, because uptake of NH_4 and acetate of different leaf litter species is negatively related. Riparian forest dominated by high quality litter such as alder and ash may provide a high-available substrate to streams, which in its turn can be less dependent on dissolved N from water column, but at the same time, more dependent on DOC from water column. In contrast, riparian forests dominated by species with leaves with low litter quality, such as sycamore, may provide streams with a poorly-available substrate, which results in a high dependence on N from water column but not for DOC. Exceptionally, riparian forests dominated by black locust could influence the strategy of microbial decomposers to obtain matter and nutrient resources (i.e., trophic strategy). Black locust could act as

colonizing substrate holding the activity of microbial decomposers which strongly rely from in-stream solute dynamics.

CHAPTER 8: RESPONSES OF MICROBIALLY DRIVEN LEAF LITTER DECOMPOSITION TO STREAM NUTRIENTS DEPEND ON LITTER QUALITY

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8.1. Abstract

The present study aims to understand how microbial decomposition of leaf litter from two riparian tree species differing in their quality varies among streams covering a gradient of nutrient concentrations. We incubated leaf litter from alder (*Alnus glutinosa*) and sycamore (*Platanus x hispanica*) in 3 streams with low human pressure and 2 streams influenced by wastewater treatment plant effluents. We quantified leaf litter decomposition rates (*k*) and examined the temporal changes in the leaf litter concentrations of carbon (C) and nitrogen (N) throughout the incubation period. We measured the extracellular enzyme activities involved in degradation of C (i.e., cellobiohydrolase) and organic phosphorus (i.e., phosphatase). Results showed that alder *k* decreased with increasing nutrient concentrations, while sycamore decomposed similarly among streams. For both species, leaf litter N concentrations were positively related to in-stream dissolved N concentrations. However, we found different temporal patterns of leaf litter N concentrations between species. Finally, we found relevant differences in the enzymatic activities associated to each leaf litter species across the nutrient gradient. These results suggest that the intrinsic characteristics of the leaf litter resources may play a relevant role on the microbially-driven leaf litter decomposition and mediate its response to dissolved nutrient concentrations across streams.

8.2. The influence of leaf litter quality and stream nutrients of leaf litter decomposition

Decomposition of leaf litter is a fundamental process in streams since it contributes to the metabolism (Webster and Benfield, 1986; Tank and Webster, 1998; Wallace et al. 1999), nutrient cycling (Tank et al. 2000), and food webs (Fisher and Likens 1973; Vannote et al. 1980) of these ecosystems. Microbial assemblages (mainly fungi and bacteria) in streams can use leaf litter as a colonizing substrate as well as a source of carbon (C) and nutrients for their development and metabolic activity. In addition, microbial assemblages on leaf litter can also meet their nutrient demand from dissolved compounds in the stream water column (Suberkroop and Chauvet 1995; Gulis and Suberkroop 2003). Therefore, both leaf litter quality and nutrient concentrations in streams are expected to influence microbial growth and activity on decomposing leaf litter, which ultimately can dictate their decomposition rates (Webster and Benfield 1986; Gulis and Superkropp 2003).

Quality of leaf litter is commonly assessed by its elemental composition (i.e., the concentration of C, nitrogen [N] and phosphorus [P]), and the relative proportions among these elements (Melillo et al. 2001). In general, leaf litter with high N and P concentration relative to C concentration decomposes faster than leaf litter with low relative concentration of N and P (Webster and Benfield 1986; Enriquez et al. 1993). Other indicators of leaf litter quality are related to the toughness of the leaves, the presence of wax products, and the complexity of organic C molecules that constitute the leaves (Webster and Benfield 1986). Simple organic compounds in leaf litter, such as soluble polysaccharides, are labile C sources; and thus, are easily degraded and consumed by microbes. In contrast, more complex C compounds in leaf litter, such as

lignin or tannins, are recalcitrant C resources; and thus, metabolically more costly to be used by microbes (Sinsabaugh et al. 1993). Therefore, relatively higher proportions of recalcitrant C sources in leaf litter have been negatively related to leaf litter decomposition rates (Schindler and Gessner 2009).

Extracellular enzyme production is the primary mechanism by which fungi and bacteria degrade polymeric and macromolecular compounds from organic matter into low-molecular-weight (LMW) molecules. LMW molecules can then be assimilated by microbial communities (Rogers 1961). In this sense, microbial activity associated with decomposing leaf litter is commonly assessed by extracellular enzyme activities (Sinsabaugh et al. 1994; Romaní et al. 2006). The most relevant extracellular enzyme activities involved in leaf litter decomposition are those related to the degradation of cellulose (such as β-glucosidase and cellobiohydrolase), hemicellulose (such as β-xylosidase), and lignin (such as phenol oxidases). In addition, N-and P-containing organic compounds are degraded by the activities of peptidases and phosphatases, respectively (Sinsabaugh et al. 1993; Romaní et al. 2006). The activity of these extracellular enzymes can be also influenced by the nutrient availability and the relative proportions between nutrients in the stream, since these enzymes can also degrade compounds from the water column (Sala et al. 2001; Romaní et al. 2004, 2012; Sabater et al. 2005; Romaní et al. 2012).

Inorganic nutrients from the water column can be additional sources of energy and matter to microbial assemblages on leaf litter (Suberkroop and Chauvet, 1995; Hall and Meyer, 1998; Ferreira et al.2015). Therefore, differences in dissolved nutrient concentrations could explain part of the observed variability in decomposition rates for

a given leaf litter type across streams (Webster and Benfield, 1986; Woodward et al. 2012). The stimulation of leaf litter decomposition by nutrient concentrations has been observed in response to increasing concentrations of dissolved inorganic N (DIN) (Richarson et al. 2004), P (Rosemond et al. 2002), and combined enrichment of N and P (Gulis and Superkropp 2003; Rosemond et al. 2015). In contrast, other studies reported that decomposition rates were not stimulated by nutrient enrichment, especially when background nutrient concentrations (i.e., before the nutrient enrichment) were not limiting (Royer and Minshall 2001; Chadwick and Huryn, 2003; Albelho and Graça, 2006; Baldy et al. 2007). Furthermore, leaf litter decomposition rates can be lowered in polluted streams, probably because other factors may counteract the stimulating effects of nutrient enrichment on leaf litter decomposition (Webster and Benfield 1986; Pascoal and Cássio 2004; Woodward et al. 2012). The relationship between microbially-driven leaf litter decomposition rates and nutrient concentrations has been also described by Michaelis-Menten models (Gulis et al. 2006; Pereira et al. 2016) suggesting that other factors beyond the nutrient concentrations may limit leaf litter decomposition rates in streams. Moreover, contrasting results among studies examining the effect of nutrient concentrations on leaf litter decomposition could be also explained by leaf litter quality, which may dictate the strength of interactions between microbial assemblages and dissolved nutrients. In this sense, a recent meta-analysis showed that the magnitude of the nutrient enrichment effect on leaf litter decomposition was usually higher for leaf litter with low and intermediated N concentrations such as *Quercus* than for high-N litter such as *Alnus* (Ferreira et al. 2015). However, in other cases the decomposition of nutrient-poor *Fagus* or *Eucalyptus* leaf litter was not affected by nutrient enrichment, suggesting that other factors beyond the litter N concentration may influence the effect of nutrient enrichment on leaf litter processing in streams (Ferreira et al. 2015).

The present study aims to understand how microbially-driven decomposition of leaf litter from two riparian tree species differing in elemental composition (i.e., C:N ratio), varies among streams which cover a gradient of nutrient concentrations. To approach this question, we incubated leaf litter from alder (Alnus glutinosa, low C:N ratio) and sycamore (Platanus x hispanica, high C:N ratio) in 5 different streams. In each stream, we assessed leaf litter decomposition rates, leaf litter C and N concentrations throughout the decomposition period, and microbial extracellular enzyme activities of cellobiohydrolase (*cbh*) and phosphatase (*phos*) after 85 d of leaf litter incubation. We expected a) that leaf litter decomposition rates would increase with nutrient concentrations, and b) to find a larger effect of nutrient concentrations on decomposition for the low-quality leaf litter species (i.e., sycamore) if nutrients in the water column act as an important additional energy and matter sources to microbial assemblages developing on leaf litter.

8.3. Methods

Study Sites

This study was performed in 5 streams located in different tributaries of La Tordera catchment (Catalonia, NE Spain, Table 8.1.). Three of them are streams with low human influence (Llavina-LLAV, Santa Fe-SF, and Font del Regàs-FR; Table 8.1.); and thus, are characterized by relatively low nutrient concentrations (von Schiller et al.2008). The other 2 streams (Gualba-GUAL and Santa Coloma-COL; Table 8.1.), receive the inputs from wastewater treatment plants (WWTP); and thus, these streams have higher nutrient concentrations. In these streams, nutrient enrichment could potentially enhance leaf litter decomposition rates. However, in many cases WWTP effluents also contain other

pollutants such as barium or aluminum, that may have the opposite effect on leaf litter decomposition (Pascoal and Cássio 2004; Woodward et al. 2012). All the study sites are 2^{nd}-3^{rd} order streams, with relatively well-preserved stream channel morphology characterized by riffles and pools. All the streams are flanked by riparian forest dominated by alder (Alnus glutinosa (L.) Gaertn.), black poplar (Populus nigra L.) and sycamore (Platanus x hispanica (Mill.) Münchh), except the SF stream where European beech (Fagus sylvatica L.) dominates the catchment as well as the stream banks.

Field experiments

For this study, we used leaves of alder and sycamore as species with high and low quality in terms of C:N ratio, respectively. Leaves from alder and sycamore were collected in November 2010 at GUAL site. To measure litter decomposition rates (*k*, degree $days^{-1}$) we followed procedures by Webster and Benfield (1986). For each leaf litter species, 5 g of air dried leaves were placed in 250 µm mesh-size bags, which mostly excluded macroinvertebrates and thus basically allowed measurement of microbial leaf litter decomposition. Leaf bags were deployed in the selected streams, anchored on the streambed with metal bars, and incubated in the streams from the 11^{th} November 2010 to the 10^{th} March 2011. At each stream, three leaf bags for each leaf litter species were collected on days 8, 15, 29, 47, 85, and 119 after deployment. Collected leaf bags were kept cold (~4ºC) in the field and in the laboratory until later measurements of dry weight and C and N leaf litter concentrations. On each sampling date, stream water samples were collected to analyze the concentrations of ammonium ($N\text{-}NH_4^+$), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), and soluble reactive phosphorus (SRP). We also measured stream discharge based on cross-section measurements of width, water depth and water velocity (Gordon et al. 2004). At each stream, we continuously

recorded water temperature every 20 minutes during the entire incubation period using temperature data-loggers (HOBO Pendant® UA-002-64) placed on the streambed. After 85 d of leaf litter incubation in the streams, we collected additional leaf bags to quantify the extracellular enzyme activities of cellobiohydrolase (*cbh*; EC 3.2.1.91) and phosphatase (*phos*; EC 3.1.3.1-2) as outlined in Romaní et al. (2006). We measured *cbh* activity as an indicator of leaf litter microbial degradation activity and especially for a recalcitrant compound such as cellulose. We measured *phos* activity to assess how changes in the inorganic nutrient availability (i.e., SRP) may affect the potential microbial use of organic phosphorus compounds. We quantified the enzyme activity after 85 d of incubation when the leaf litter packs roughly loosed 40–60% of initial mass. At this point, we expected that microbial assemblages were well developed and extracellular enzyme activities were high (Romaní et al. 2006).

Laboratory methods and data analysis

Stream water samples were analyzed at the Nutrient Analysis Service of the Centre d´Estudis Avançats de Blanes (CEAB) for nutrient concentrations using an Automatic Continuous Flow Futura-Alliance Analyzer and following standard colorimetric methods (APHA, 1995).

In the laboratory, leaf litter samples collected on each sampling date and at each stream were carefully rinsed with stream water to remove inorganic sediment attached to the leaf surface. Then, leaf litter samples were oven-dried until constant weight (60 ºC for 48 hours) and weighed to obtain the remaining dry mass. Sub-samples of leaf litter were ignited (500 ºC, 4 hours) to calculate ash-free dry mass (AFDM), which was expressed as percentage of the initial AFDM. The remaining AFDM on each sampling date for each leaf litter types and for each stream was plotted against degree-days (i.e. summing

the daily mean temperature registered along the study period). The relationship fitted a negative exponential model described by Petersen and Cummins (1974)

$$W_t = W_0 * e^{-k\,dd} \quad (1)$$

where W0 and Wt are AFDM (g) at the beginning and at sampling dates, respectively; dd (degree-days) is the incubation time expressed in terms of summed mean daily water temperature (ºC) up to the sampling dates and *k* is the decomposition rate (expressed in terms of dd^{-1}). Values of *k* denote the velocity at which mass of leaf litter decreases over time corrected for the potential temperature differences among streams, so that *k* values can be compared among sites with different water temperatures.

Concentration of C (g C/g DM) and N (g N/g DM) in leaf litter before and over the incubation period for the 2 leaf litter species and among the 5 study streams were measured for the collected samples. Dried sub-samples were ground to a fine powder, and a sub-sample of 1.5 mg was weighed and encapsulated in tin vials. Samples were sent to the Unidade de Técnicas Instrumentais de Análise (Universidade da Coruña, Spain) for the analysis of elemental C and N concentrations, which was done by sample combustion using an elemental autoanalyzer EA1108 (Carlo Erba Instruments). Data of N concentrations at d 85 was used to explore how the effect of dissolved nutrient concentrations influences on leaf litter N concentrations.

Extracellular enzyme activities of *cbh* and *phos* on leaf litter samples incubated for 85 d were measured using methylumbelliferyl (MUF) fluorescent-linked substrates, following the method described in Romaní et al. (2006). These assays were conducted at saturation substrate conditions of 0.3 mM. Leaf litter discs (14 mm diameter, 3 replicates per experimental condition) and water controls were incubated for 1 h in the

dark in a shaker. Blanks and standards of MUF (0–100 µmol L^{-1}) were also incubated. At the end of the incubation, Glycine buffer (pH 10.4) was added (1/1 vol/vol), and the fluorescence was measured at 365/455 nm excitation/emission (Kontron SFM25 fluorimeter). Results of extracellular enzyme activities were expressed as the amount of MUF substrate produced per incubation time (h) and leaf litter ash free dry mass (AFDM; g).

Statistical analysis

To determine differences in the physical and chemical variables among study streams, we used a one-way analysis of variance (ANOVA) model with stream (n=5) as fixed factor followed by Post-hoc Tukey-t test. We also used a one-way ANOVA model to determine initial differences in the leaf litter C and N concentrations and the C:N ratio among the 2 leaf litter species.

We used a two-way ANCOVA to explore differences in leaf litter *k* between the 2 leaf litter species and among the 5 study streams. Fraction of litter remaining AFDM of alder and sycamore was natural log transformed prior to the analysis. The two-way ANCOVA included fraction remaining AFDM as dependent variable, time (expressed in degree-days) as the covariate and stream (n=5) and leaf litter species (n=2) as fixed factors. We used the interaction term stream*species*degree-days to explore the null hypothesis in which the variability in *k* among streams did not differ among leaf litter species (Zar, 1999). Additionally, to explore the specific variability of *k* for each leaf litter species among streams, we also used a one-way ANCOVA for each leaf litter species, which included fraction remaining AFDM as dependent variable, time (expressed in degree-days) as the covariate and stream (n=5) as a fixed factor. Tukey´s test followed significant differences among streams.

To examine differences in the variation in the leaf litter C and N concentrations during the leaf litter decomposition between leaf litter species and across streams, we used two-way ANOVA with repeated measures (RM, i.e., sampling time) with both leaf litter C and N concentrations as dependent variables, respectively; leaf litter species (n=2) and streams (n=5) as fixed factors and time (expressed in days) as the covariate. In addition, we used linear and asymptotic-type models to explore the best fit of the temporal variation in the N concentrations throughout decomposition period of leaf litter for both alder and sycamore (from 11th November 2010 to the 10th March 2011).

The asymptotic model followed the equation:

$$N = \frac{N_{max}\ d}{K_d + d} \qquad (2)$$

Where N_{max} is the maximum leaf litter N concentrations, K_d is the incubation day at which N reach the half of N_{max} concentrations and d is the incubation time (in days).

We examined differences in extracellular enzyme activities of both *cbh* and *phos* using a two-way ANOVA model with stream (n=5) and leaf litter species (n=2) as fixed factors. We used Pearson correlation coefficients (PCC) to explore relationships between *cbh* and *phos* activities on each leaf litter species. In addition, we explored the relationships between both, *cbh* and *phos* extracellular enzyme activities and the percentage of leaf litter mass loss among streams using data from the d 85 of leaf litter incubation. To do that, we used linear, exponential and asymptotic relationships in order to find the best-fit model.

Finally, to assess differences between leaf litter species in terms of *k*, leaf litter N concentrations, and *cbh* and *phos* activities across increasing nutrient gradient, we explore linear relationships between these parameters and the concentrations of DIN and SRP and the DIN:SRP molar ratio of the study streams for the 2 leaf litter species separately.

Statistical analyses were done with PASW Statistics 18 (v18.0.0/SPSS Inc) and R 2.14.0 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.). Statistical results were evaluated at the $\alpha = 0.05$ significance level.

8.4. Results

Stream characteristics

Stream discharge varied among streams, and was lower in SF and FR than LLAV and the two streams influenced by WWTP effluents (GUAL and COL) (Table 8.1.). Mean water temperature varied 4 ºC among streams, and was higher in GUAL and COL streams and lowest in SF, the stream located at the highest elevation (Table 8.1.). DIN and SRP concentrations covered a wide range among streams, especially for the DIN species, which spanned two orders of magnitude (Table 8.1.1). Concentrations of DIN and SRP were strongly correlated among streams (PCC, r = 0.90, p <0.001) and both were higher in the streams influenced by WWTP inputs (Table 8.1.). The concentration of NO_3 accounted for the largest fraction of the DIN concentration in all the streams; however the percentage of DIN as NH_4 was higher in the streams influenced by WWTP inputs (Table 8.1.).

Table 8.1. Longitudinal (Long.) and latitudinal (Lat.) location of the streams, average and SEM (in parenthesis, n=21) of physical and chemical variables for each stream during the study period, decomposition rates (*k*) for alder and sycamore and the ratio between decomposition rates of both alder and sycamore leaf litter. Different letters indicate significant differences on *k* based on one-way ANCOVA analysis and in the rest variables based on ANOVA analysis, followed by post-Hoc Tukey´s t-test. Note that for *k* capital and lower case letters indicate statistical differences among streams and between leaf litter species, respectively. DIN= dissolved inorganic nitrogen (nitrite + nitrate + ammonia). Streams influenced by wastewater treatment plant inputs are indicated with asterisks.

Long. 2ºE	Lat. 41ºN	Discharge ($L\ s^{-1}$)	Temp. (ºC)	NO_3 ($\mu g\ N\ L^{-1}$)	NH_4 ($\mu g\ N\ L^{-1}$)	SRP ($\mu g\ P\ L^{-1}$)	DIN ($\mu g\ N\ L^{-1}$)	*k* alder (dd^{-1})	*k* sycamore (dd^{-1})	*k* alder : *k* sycamore ratio
27´52´´	46´37´´	67 (29)^{a}	5.3 (0.1)^{a}	39 (13)^{a}	13 (3)^{a}	13 (2)^{a}	51 (13)^{a}	0.00132 $^{A-a}$	0.00085 $^{A-a}$	1.55
27´00´´	49´32´´	67 (14)^{a}	6.5 (0.2)^{b}	150 (26)^{ab}	19 (4)^{a}	5 (1)^{a}	169 (27)^{ab}	0.00131 $^{A-a}$	0.00066 $^{A-b}$	1.98
23´52´´	45´09´´	224 (113)^{b}	6.7 (0.1)^{ab}	261 (47)^{ab}	27 (8)^{a}	9 (1)^{a}	288 (45)^{b}	0.00148 $^{A-a}$	0.00067 $^{A-b}$	2.21
30´17´´	44´02´´	155 (33)^{b}	7.3 (0.2)^{ab}	307 (40)^{b}	471 (8)^{b}	75 (12)^{ab}	778 (100)^{c}	0.00093 $^{A-a}$	0.00058 $^{A-a}$	1.60
39´32´´	51´48´´	156 (37)^{b}	9.4 (0.2)^{b}	1549 (127)^{c}	941 (288)^{c}	103 (47)^{b}	2490 (224)^{d}	0.00064 $^{B-a}$	0.00053 $^{A-a}$	1.21

Initial leaf litter C and N concentrations and leaf litter decomposition rates

Alder and sycamore leaf litter presented similar C concentrations (44.65±0.56 and 44.60±0.45 % of dry mass, respectively) (one-way ANOVA, $p > 0.05$). However, alder showed higher N concentrations than sycamore (2.03±0.09 and 1.32±0.12 % of dry mass, respectively) (one-way ANOVA, $p < 0.001$). Therefore, the C:N ratio of alder leaf litter was significantly lower than the C:N ratio of sycamore leaf litter (one-way ANOVA, $p < 0.001$).

On average, *k* values of alder leaf litter were higher than *k* values of sycamore leaf litter (two-way ANCOVA, Tukey-t test, $p < 0.001$, Table 8.1.). The variability in *k* values among streams was higher for alder than for sycamore leaf litter (Table 8.1.). Among streams, *k* values for both alder and sycamore leaf litter were lower in streams influenced by inputs from WWTP effluents (two-way ANCOVA, Tukey-t test, $p < 0.001$, Table 8.1.). In addition, in COL (i.e., the stream with the highest nutrient concentrations) we found a smaller difference in *k* between the two leaf litter species (*k* alder : *k* sycamore = 1.21; Table 8.1.). Overall, *k* rate for alder leaf litter was negatively related to stream DIN concentrations ($r^2=0.77$, $p < 0.001$, Figure 8.1. A and Table S8.1.; see annexes section) and SRP concentration ($r^2=0.93$, $p < 0.001$, Table S8.1. see annexes section). In contrast, no relationships were found between *k* values for sycamore leaf litter and DIN and SRP concentrations ($p > 0.05$, Figure 8.1. B and Table S8.1; see annexes section). Leaf litter *k* was not related with DIN:SRP molar ratio among streams for neither leaf litter species (Table S8.1.; see annexes section).

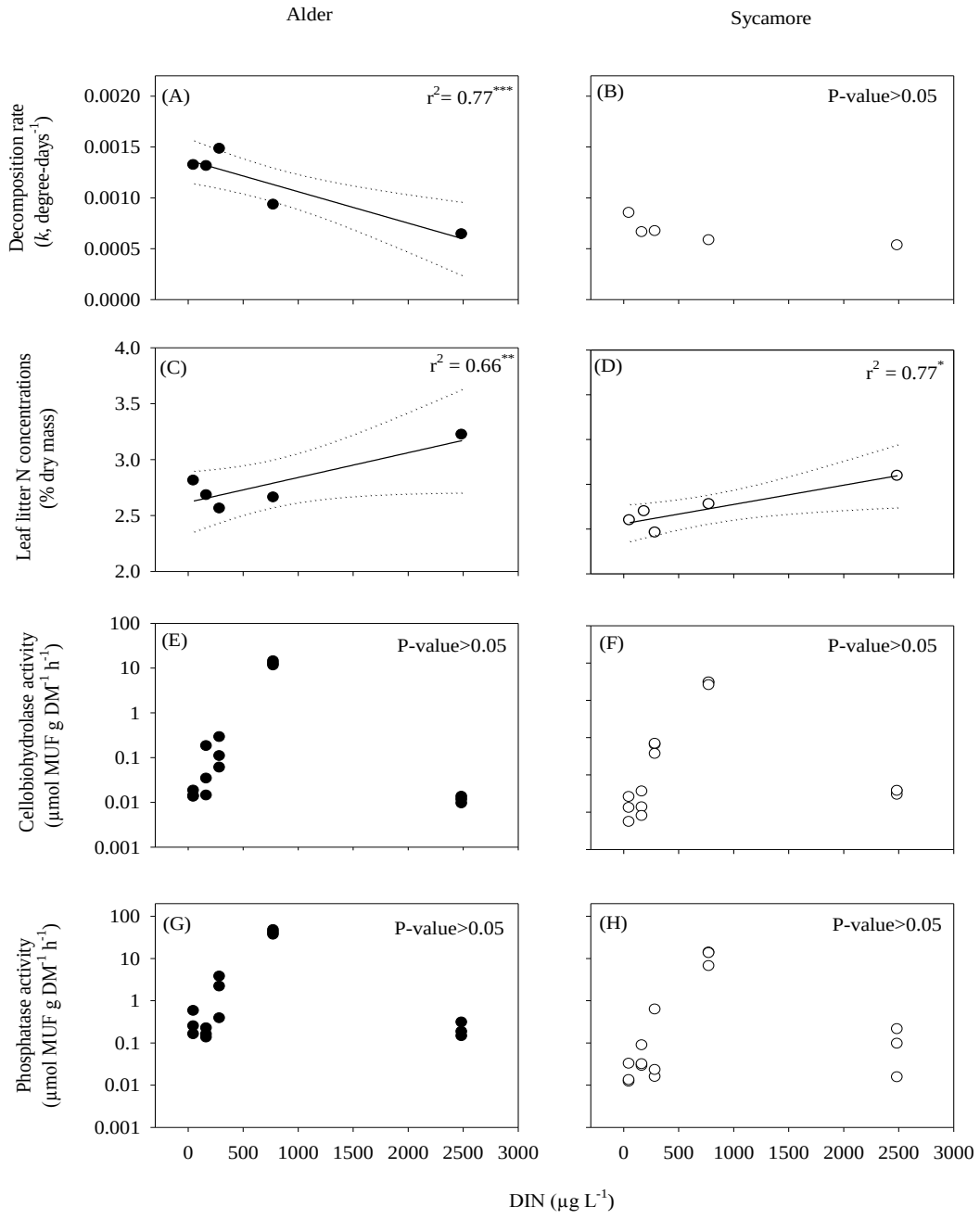

Figure 8.1. Relationships between in-stream DIN concentrations and leaf litter decomposition rates (A-B), the leaf litter N concentrations measured at exposure time of 85 d (C-D), and the extracellular enzyme activities of both cellobiohydrolase and phosphatase measured at exposure time of 85 d (E-H). Filled circles (left panels) and open circles (right panels) correspond to data of alder and sycamore leaf litter. Level of significance based on one-way ANOVA analysis is indicated by: *** P-value<0.001 ** P-value<0.01 and * P-value<0.05. DIN= dissolved inorganic nitrogen (nitrite + nitrate + ammonia).

Variation in leaf litter C and N concentrations during the decomposition period

The C concentrations did not significantly vary during decomposition period, and values were similar among leaf litter species and among streams (ANOVA-RM, $p > 0.05$). In contrast, the N concentrations differed among leaf litter species (ANOVA-RM, $p < 0.01$), with alder leaf litter showing higher N concentrations than sycamore leaf litter. The N concentrations of leaf litter during the decomposition period varied among streams (ANOVA-RM, $p < 0.01$), with highest values in COL and lowest values in LLAV. The interaction term (i.e., leaf litter species*stream) of the ANOVA-RM was not significant ($p > 0.05$) indicating that differences in N concentrations between alder and sycamore leaf litter during the decomposition period were consistent among streams. The leaf litter N concentrations at d 85 of incubation period was positively related to stream DIN concentrations for both alder and sycamore leaf litter ($r^2=0.66$, $p < 0.01$, $r^2=0.77$, $p < 0.05$, respectively, Figure 8.1.C and 8.1. D and Table S8.1; see annexes section).

The temporal patterns of N concentrations during the decomposition period differed between alder and sycamore leaf litter. The temporal variation of N concentrations in alder leaf litter was best fitted with an asymptotic-type model in all streams (Figure 8.2., left panels), except in LLAV (Figure 8.2. E). N concentrations showed a rapid increase during the early stages of the leaf litter decomposition but then reached a steady state until the end of the incubation period. In contrast, the temporal variation of N concentrations in sycamore leaf litter during the incubation period followed a linear model in all streams (Figure 8.2., right panels), except in GUAL (Figure 8.2. H).

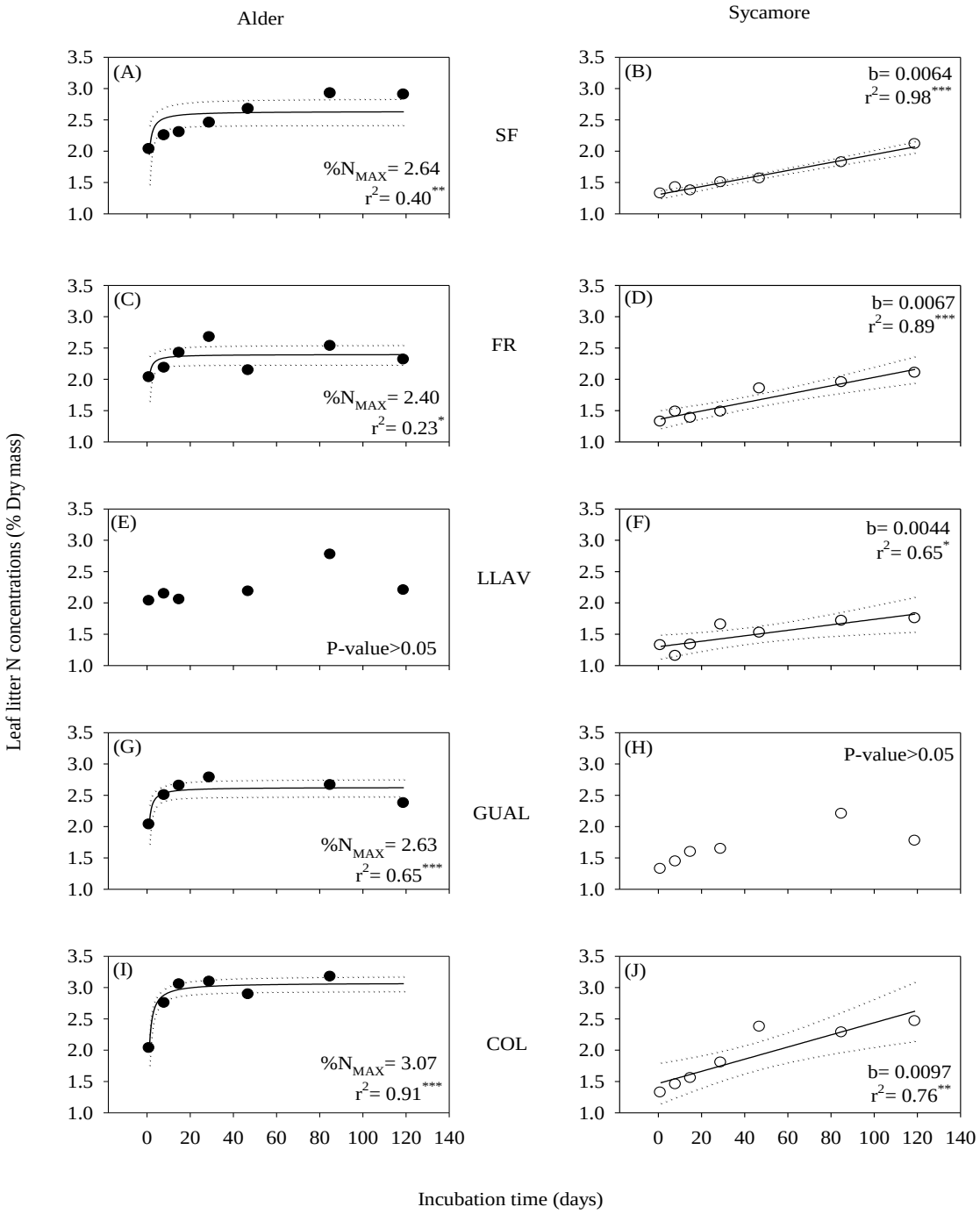

Figure 8.2 Temporal variation in the leaf litter N concentrations (as percentage of dry mass) for alder (left panels; asymptotic-type models) and sycamore (right panels; linear models) during the decomposition period in the 5 studied streams. Filled circles (left panels) and open circles (right panels) correspond to data of alder and sycamore leaf litter. N_{max} is the maximum N concentrations on leaf litter during decomposition period (left) and b is the slope of the linear model (right). Level of significance of the models is indicated by: *** P-value<0.001, ** P-value<0.01 and * P-value<0.05.

Extracellular enzyme activities

The extracellular enzyme activity of *cbh* was higher for alder than for sycamore leaf litter (2.97 ± 1.6 and 0.57 ± 0.29 µmol MUF g DM^{-1} h^{-1}, respectively; ANOVA, $p < 0.001$; Figure 8.3. A). Values of *cbh* for both alder and sycamore leaf litter significantly differed among streams (ANOVA, $p < 0.001$; Figure 8.3. A). Basically, the higher *cbh* activities for the two leaf litter species were measured in streams with intermediate nutrient concentrations (i.e., LLAV and GUAL). The interaction term of the ANOVA (leaf litter species*stream) was not significant ($p > 0.05$), indicating that the variation in *cbh* among streams was consistent among leaf litter species.

Extracellular enzyme activity of *phos* was higher for alder leaf litter than for sycamore leaf litter (8.73 ± 4.33 and 2.30 ± 1.24 µmol MUF g DM^{-1} h^{-1}, respectively; ANOVA, $p < 0.001$; Figure 8.3. B). Values of *phos* for both alder and sycamore leaf litter significantly differed among streams (ANOVA, *p* 0.001; Figure 8.3. B), and the interaction term (leaf litter species*stream) was not significant (ANOVA, $p > 0.05$). Extracellular enzyme activities of *cbh* and *phos* were strongly correlated for both alder leaf litter (PCC, r = 0.97, $p < 0.01$) and sycamore leaf litter (PCC, r = 0.95, $p < 0.01$).

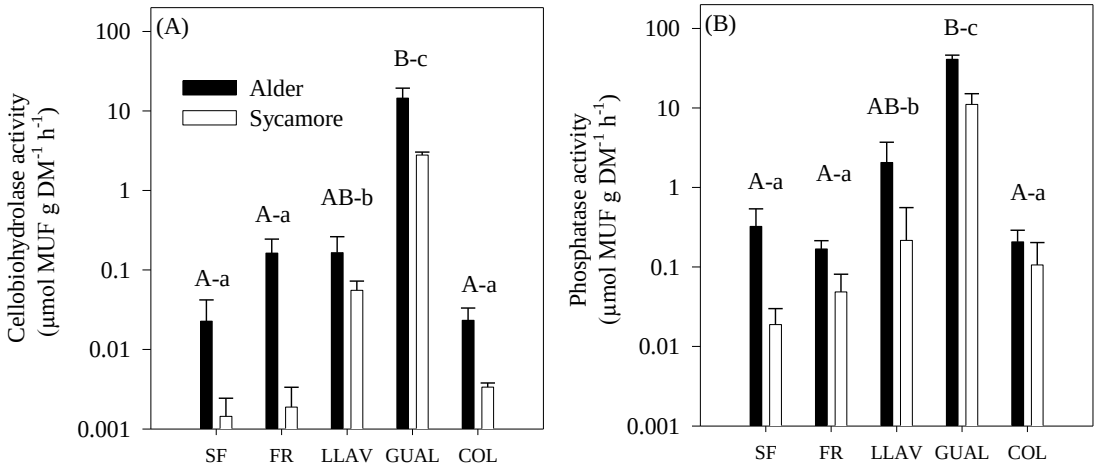

Figure 8.3. Extracellular enzyme activities of cellobiohydrolase (left) and phosphatase (right) (+SEM, n=3 per experimental condition) measured on alder and sycamore leaf litter at incubation time of 85 d. Significant differences among streams for alder and sycamore leaf litter species are shown as different capital and lower case letters, respectively, based on two-way ANOVA analysis. Note that streams are ordered following the increasing gradient of DIN concentration, being SF the stream with lowest concentration and COL the stream with the highest concentration.

Considering data from all streams together, leaf mass loss by d 85 was significantly related to both *cbh* and *phos* activity for alder leaf litter (Figure 8.4. A and 8.4. C), but it was not related to any extracellular activity for sycamore leaf litter (Figure 8.4. B and 8.4. D). Specifically, for the case of alder leaf litter, we found that the relationship between alder leaf mass loss and enzyme activities of both *cbh* and *phos* was best fitted with an asymptotic-type model (r^2 = 0.57, p < 0.001 and r^2 = 0.78, p < 0.001, respectively, Figure 8.4. A and 8.4. C).

Activities of both *cbh* and *phos* did not correlated with concentrations of DIN, SRP nor the DIN:SRP molar ratio among streams (p > 0.05, Figure 8.1. E-H and Table S8.1.; see annexes section). Nevertheless data showed a hump-shape trend characterized by an initial increase of enzyme activities up to 1 mg L^{-1} of DIN followed by a clear decrease above this threshold (Figure 8.1. E-H).

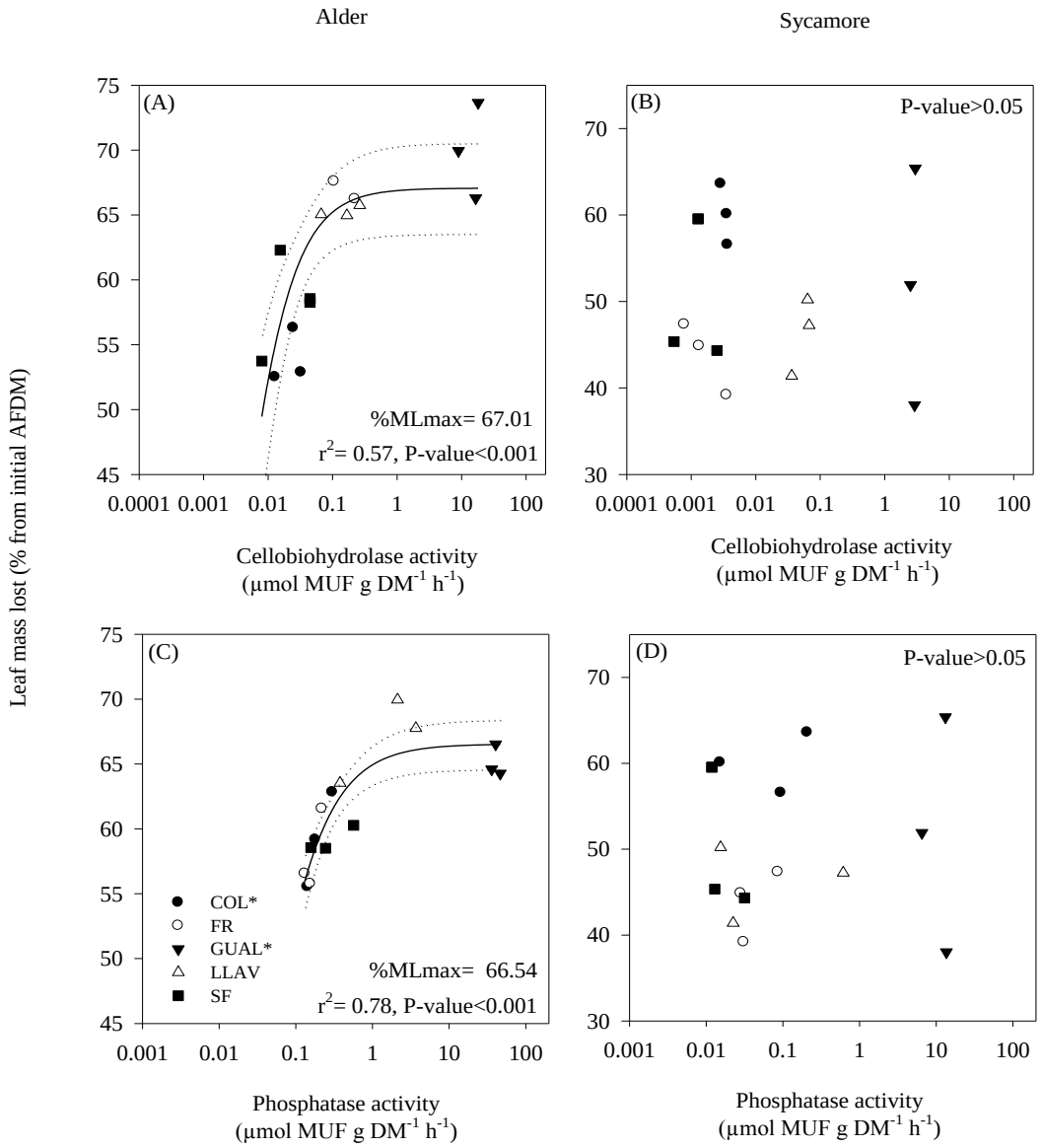

Figure 8.4. Relationships between the percentage of leaf litter mass lost on 85 d of leaf litter incubation and the microbial activities of both cellobiohydrolase (up panels) and phosphatase (down panels) for the two leaf litter species, considering data from all streams together. Data from alder leaf litter (left panels) was best fitted by an asymptotic-type model, where %MLmax is the maximum alder mass lost among streams from the model.

8.5. Discussion

The influence of nutrient gradient on leaf litter decomposition rates

We found that the response of microbially-driven leaf litter decomposition rates to the stream nutrient gradient differed between the two leaf litter species considered. This agrees with previous finding (Ferreira et al. 2015) and reinforces the notion that leaf litter quality mediates the responses of leaf litter decomposition to dissolved nutrient concentration in streams. Nevertheless, results do not agreed with our expectations since decomposition rates of alder decreased along the nutrient gradient, while no significant changes were observed in decomposition rates of sycamore across the nutrient gradient. These results suggested that decomposition of high-quality leaf litter (i.e., low C:N ratio), such as alder, may be more sensitive to differences in nutrient concentrations among streams than low-quality leaf litter, such as sycamore. In this sense, Woodward et al. (2012) also found higher variability on decomposition rates for high-quality leaf litter species such as alder than for low-quality litter such as oak across streams covering a 1000-fold nutrient gradient. However, in contrast to our results, their observed responses to increased nutrient concentrations exhibited a hump-shape pattern. Nevertheless it is worth noting that in Woodward et al. (2012) the significant hump-shape pattern was only observed on total decomposition which includes macroinvertebrate leaf litter breakdown. Other studies focusing on microbial decomposition also observed a lack of response of *k* across stream nutrient gradient (Chauvet et al. 2016). Overall these results suggest that other factors beyond nutrient concentrations may influence microbial-driven decomposition rates across streams. In this sense, in a recent study conducted under laboratory conditions, Fernandes et al. (2014) found that Michaelis-Menten kinetics best explained the relationship between microbial-driven leaf litter decomposition rates and N availability, suggesting that the

activity of microbial assemblages colonizing leaf litter become limited by other factors when N availability in streams increases as outlined in Bernot and Doods (2005).

We found that microbially-driven decomposition of alder was lower in highly polluted streams although it has been reported that nutrient enrichment had a positive or saturating effects on microbial biomass and activity associated with decomposing leaf litter (Suberkropp and Chauvet, 1995; Fernandes et al. 2014), as well as, on leaf litter decomposition rates (Fernandes et al. 2014; Ferreira et al. 2015; Rosemond et al. 2015). Our results agree with previous studies showing that on highly polluted streams decomposition is generally reduced regardless of the high stream nutrient concentrations (Pascoal and Cássio 2004; Lecert et al. 2006; Woodward et al. 2012). A plausible explanation of these results is that in polluted streams, such as those receiving the effluents from WWTPs, confounding factors may influence the positive effect of nutrient concentrations on leaf litter decomposition (Pascoal and Cássio 2004; Woodward et al. 2012). In fact, in our WWTP-influenced streams the relatively proportion of NH_4 with respect to total DIN concentrations was higher with respect to that in more pristine streams. A previous study found that NH_4 may inhibit leaf litter decomposition rates (Lecert et al. 2006). Furthermore, WWTP effluents are sources of other compounds such as metals and emergent pollutants, which may have negative effects on the microbial communities, as well as, on leaf litter decomposition rates (Webster and Benfield, 1986; Pascoal and Cássio, 2004; Ferreira et al.2016). Thus, in WWTP-influenced streams these factors could potentially counterbalance the positive effects of nutrient enrichment on leaf litter decomposition leading to the decrease of organic matter decomposition (Kaushik and Hynes 1971; Pascoal and Cássio 2004; Woodward et al. 2012).

Differences between leaf litter species during the decomposition period

Decomposition rates of alder leaf litter were consistently higher than those of sycamore leaf litter, regardless of the stream, suggesting that the intrinsic characteristics of the leaf litter may also drive to some extend *k*. This pattern may be related to the higher N concentration, as well as, low concentration of refractory compounds such as lignin on alder leaves with respect to that of sycamore (Webster and Benfield 1986; Gessner and Chauvet 1994; Cornwell et al. 2008). Nevertheless, in this study, the differences in decomposition rates between alder and sycamore leaf litter were smaller than in other studies (Webster and Benfield 1986), which could be in part attributed to the lower C:N ratio of sycamore leaf litter (34±0.5) comparing to values reported previously (C:N = 73.6; Gessner and Chauvet 1994). Nevertheless, we found that the difference in decomposition rates between the two leaf litter species decreased among streams as nutrient concentrations and pollution conditions increased. This suggests that in polluted streams, environmental conditions seem to be more relevant than specific characteristics of the leaf litter on determining the rates of organic matter decomposition.

Alder and sycamore N concentrations at later stages of decomposition period increased as DIN concentrations in streams increased, suggesting that the availability of DIN in streams can influence the activity of microbial assemblages on leaf litter (Molinero et al. 1996, Pozo et al. 1998, Tank et al.2000; Gulis and Suberkropp 2003). This response contrasted with that observed for leaf litter decomposition, pointing that mechanisms controlling N concentrations of the microbial-leaf litter complex during the decomposition could be independent of the efficiency at which leaf litter mass is lost. However, differences between leaf litter species were highlighted by the different models describing the temporal variation of leaf litter N concentrations between species. These results suggest that, regardless of the stream conditions, leaf litter quality is a

relevant factor controlling the dynamics of microbial colonization on leaf litter. Microbial colonization may be faster in high quality leaves, such as alder, than in low quality leaves, such as sycamore. These results are in agreement with previous studies about microbial colonization patterns of leaf litter differing in nutrient concentration (Webster et al. 2009) or in the content of recalcitrant compounds (Gessner and Chauvet 1994), which are factors that can limit growth of fungi on leaf litter (Canhoto and Graça 1999).

The influence of nutrient gradient on enzyme activities

The variability of *cbh* and *phos* enzyme activities was remarkable among streams and observed patterns were consistently similar for the two leaf litter species, suggesting that water column characteristics can influence the enzymatic activity of microbial assemblages coating leaf litter. We found that *cbh* and *phos* increased as DIN concentration increased; however at DIN concentration >1mgN L^{-1} the two enzymatic activities were significantly depressed. *Cbh* and *phos* are catabolic enzymes, and their expression can be regulated by organic compounds from the leaf litter as well as by chemical compounds from stream water column (Sala et al. 2001, Romaní et al. 2004, 2012). In fact, Sinsabaugh et al. (2005) found that increases in DIN availability lowered *cbh* activity in leaf litter, which is to some extent, in agreement with our results. A similar trend was also found for stream water SRP availability and *phos* activity (Romaní et al. 2004, 2012; Allison and Vitousek 2005). Overall, these results suggest that enzymatic responses depend on the nutrient availability. In addition, other compounds such as pollutants coming from the WWTPs inputs could also affect extracellular enzyme activities of microbial assemblages (Webster and Benfield 1986; Freeman and Lock, 1992). In COL, the presence of these compounds could have

lowered the *cbh* and *phos* activities and by extension the decomposition rates (Pascoal and Cássio 2004; Woodward et al. 2012).

The activity associated to cellulose and organic phosphorus decomposition was consistently lower in microbial assemblages growing on sycamore leaf litter than in those growing on alder leaf litter. This pattern also supports the clear effect of leaf litter quality on the activity of the microbial assemblages decomposing organic matter. This agrees with previous studies showing lower values of *cbh* activity in sycamore leaf litter in comparison to alder leaf litter (Artigas et al.2004) or other nutrient rich leaf litter species such as black poplar (Artigas et al.2011). Other studies have attributed the lower values of enzyme activities in sycamore to the higher lignin and tannin concentration of these leaves (Gessner and Chauvet, 1994).

We found that enzyme activities were related with leaf litter mass loss only for alder. This result suggests that leaf litter quality could regulate the enzyme efficiency involved in the leaf litter mass loss across streams. Nevertheless, the highest values of both activities observed in GUAL stream were not related to higher mass loss on alder. In this stream, microbial enzymatic activity could be fueled by a combination of leaf litter resources and water column nutrients, which may explain why the increasing of microbial activity did not result in a stimulation of leaf litter mass loss (Suberkroop and Chauvet 1995). In contrast, the weak relationship between enzyme activities and mass loss in sycamore leaf litter suggested that other enzymes, such as phenol oxidases, may be a limiting step for the decomposition of the leaf tissues. Overall, these findings suggest that enzymatic activity of *cbh* and *phos* of microbial assemblages developing on sycamore leaf litter could be also fueled by dissolved organic sources from water column. Additionally, results suggest that the decomposition of sycamore leaf litter is

more limited by the quality of this leaf litter than by the availability of external resources.

Alder and sycamore leaf litter consistently showed different decomposition rates, temporal dynamics of leaf litter N concentrations and enzyme efficiency of microbial decomposers across the stream nutrient gradient. These results suggest that the influence of stream environmental characteristics on particulate organic matter decomposition may depend on the quality of leaf litter where microbial assemblages develop. Nevertheless, our study suggests that stream characteristics can also negatively influence organic matter decomposition, especially in those streams affected by pollution from WWTP effluents. Overall, the present study suggests that the riparian species composition may play a relevant role on leaf litter decomposition in streams. However, this role could be less clear in polluted streams such us those receiving inputs from WWTPs where leaf litter decomposition and associated microbial activity seems to be inhibited. In conclusion, vegetation with high quality leaf litter (i.e., alders) dominating riparian forest could provide a more bioavailable leaf litter substrate for in-stream microbes. In contrast, vegetation with low quality leaf litter (i.e., sycamore) may provide a less bioavailable decomposing substrate for microbial assemblages, which could grow and develop their enzymatic activity uncoupled to leaf litter mass loss and thus, to the dynamics of organic matter decomposition across streams.

CHAPTER 9: GENERAL DISCUSSION

Understanding the relevance of leaf litter to streams involves the assessment of leaf litter dynamics (i.e., leaf litter inputs, export, and processing) (Wallace and Webster, 1995), as well as, how leaf litter interacts to stream water column (Webster and Benfield, 1986; Tank et al. 2000; Sobota et al. 2012; Tank et al. 2017) (Figure 1.1.). This thesis indicates that the heterogeneity of water velocity within a reach can influence leaf litter dynamics across different scales of observation. In particular, we found that water velocity influences the retention and spatial distribution of leaf litter at reach scale, and also how these inputs are processed at habitat scale (microbial consumption vs physical fragmentation) (chapter 5). The present thesis also shows that leaf litter quality influences the chemical an optical characteristics of leaf litter leachates, as well as, how these leachates are used by stream microbial assemblages (chapter 6). Leaf litter quality also modulates the microbial uptake of DIN and DOC from water column during leaf litter decomposition (chapter 7). Finally, our results indicate that responses of microbially driven leaf litter decomposition to stream nutrients depend on leaf litter quality (chapter 8). Considering all these results together, we suggest that stream hydro-morphology is a controlling factor of leaf litter dynamics in streams, as well as, leaf litter quality ultimately determine the interaction between leaf litter and stream water column.

9.1. Influence of stream hydro-morphology on leaf litter dynamics in streams

The present thesis demonstrates that even in low-order streams leaf litter transport and export occurs at stream baseflow. This observation contrasts with previous statements which assumes that most of the leaf litter inputs from the riparian zone are retained in receiving channels of low-order streams (Vannote et al. 1980; Snaddon et al. 1992, Raikow et al. 1995, Dewson et al. 2007) and that leaf litter export in low-order streams mainly occurs during flood events (Webster et al. 1994; Wallace et al. 1995). Therefore, we may expect that even under baseflow conditions, only a fraction of the leaf litter inputs will be effectively retained in the reach and available as a resource for stream communities (chapter 5). The present thesis also provides that the heterogeneity of water velocity within the reach may be a potential factor controlling leaf litter transport and export under baseflow conditions. In fact, it has been traditionally assumed that the export of leaf litter under baseflow conditions has been positively related to stream discharge (Webster et al. 1999), and also it has been influenced by the complexity of streambed morphology (Richardson et al. 2009). However, further studies considering the complexity of streambed morphology into empirical models of leaf litter retention/transport have been limited by the difficulty to find a good descriptor for this complexity. One way to estimate the complexity of streambed morphology is by using the Manning´s roughness coefficient ("n"), which determines the resistance of stream-water to flow through the streambed. This parameter can be easily estimated by using Manning's equation whether the average water velocity at the stream reach is known. However, this parameter calculates the average streambed roughness, overlooking the heterogeneity of the streambed. We found that the heterogeneity of water velocity within the reach could be a factor integrating the entire streambed complexity for a given reach (chapter 5). Although our results come from a single reach, they suggest

that the heterogeneity of water velocity could be used as a surrogate of the complexity of streambed morphology to predict variation of leaf litter retention among streams. For instance, stream reaches characterized by a high streambed roughness (i.e., low leaf litter export) would probably have a high heterogeneity of water velocities. In contrast, streams with a few homogeneous substrates in the streambed (i.e., high leaf litter export)we would expect a low heterogeneity of water velocities. In this context, here we validate the combined effect of stream discharge and streambed complexity estimated from an index of heterogeneity in water velocity. We use data from unpublished additions of leaves of *Ginkgo biloba* we did in 6 stream reaches (~80m length) which covered a wide gradient of stream discharge and streambed morphology (see annexes section, *assessment leaf litter export across streams*, pp. 191-192). The results obtained from these additions show a positive relationship between leaf litter export and stream discharge, which followed a logarithm model ($y = a \ln [x-x0]$; Figure 9.1. A). Leaf litter export was negatively related to the heterogeneity of water velocity (Figure 9.1. B). These results support our previous suggestions (chapter 5) because the heterogeneity of water velocity could be an important factor explaining differences on leaf litter export among streams of different hydro-morphology characteristics. Therefore, we suggest that the combination of stream discharge and the heterogeneity of water velocity (as a surrogate of streamed morphology) should be simultaneously assessed to describe leaf litter retention/export in a global context.

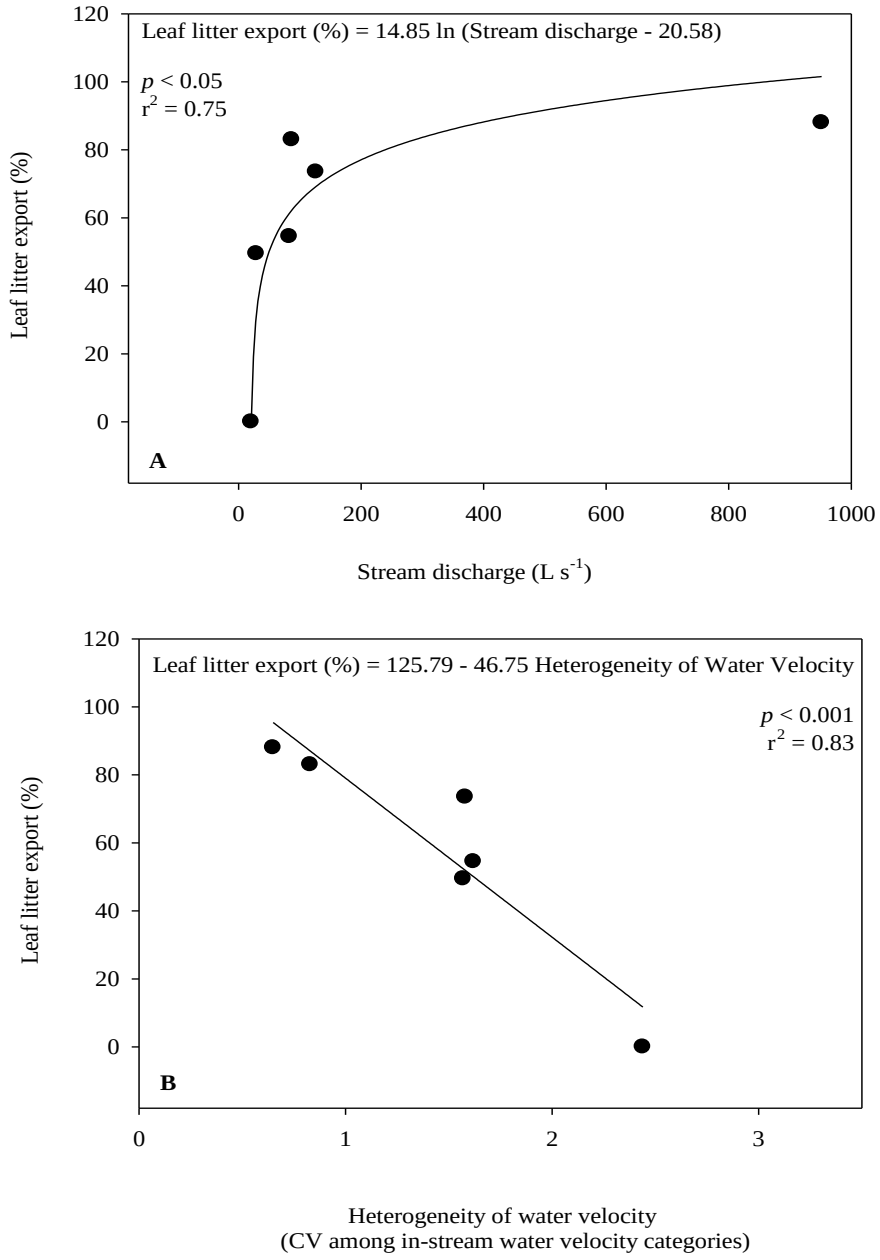

Figure 9.1. Results from leaf litter tracer additions conducted in 6 stream reaches differing in hydro-morphological characteristics (unpublished data), which show that the variation in the leaf litter export among streams is as a function of: (A) stream discharge and (B) heterogeneity of water velocity within the stream (see annex section for detailed information on how this parameter is estimated). Where: *p* and r^2 are the p-value and the coefficient of determination of the regression, respectively.

The present thesis also indicates that the heterogeneity of water velocity within a reach may influence the spatial distribution of leaves within the reach. We observed that after short-time periods of leaf litter inputs they retain near of its entry point (Snaddon et al. 1992, Raikow et al. 1995, Dewson et al. 2007) and covering a wide mosaic of water velocities. As time since leaf litter inputs increases, leaves re-distribute themselves along the reach, with a transition from high to low velocity zones. This may suggest that low velocity zones favor leaf litter retention (Hoover et al. 2006). Alternatively, the accumulation of leaves at low velocity zones could be explained by the high dominance of these zones within the reach (~70%, chapter 5). Nevertheless, we found that under mid-velocity conditions (~20 - 50 cm s^{-1}) leaves were more effectively retained than expected from the relative abundance of locations within the reach. Our study does not certainly explain the observed leaf litter distribution within the reach. However, we suggest that a combination of relatively low velocity zones, where leaves are deposited and mid-velocity areas, where leaves are effectively trapped, may explain how leaf litter inputs distribute within the reach. Our results further suggest that leaves can be effectively retained and exposed to decomposition by microbial assemblages under different ranges of water velocity, which may ultimately dictate how leaf litter is processed within the reach (microbial consumption vs physical fragmentation).

The present thesis shows that leaf litter decomposition rates (*k*) are widely variable within the reach (CV~41%; from 0.008 to 0.022 d^{-1}), and that this variability can be explained by water velocity differences among stream habitats (chapter 5). Our range of *k* values within a 80-m long reach for a given leaf litter species is similar than that described by Petersen and Cummins (1974) considering several leaf litter species. Likewise, our range of variation of *k* is even wider than that found by Woodward et al.

(2012) when they assessed the influence of stream nutrient concentrations on leaf litter *k* across streams covering a 1000-fold nutrient gradient. Therefore, the heterogeneity of water velocity may be a pivotal factor controlling leaf litter decomposition. We suggest that values of *k* provided by previous studies could have been under/overestimated because they ignore the velocity conditions during leaf litter decomposition. Previous studies up-scale at ecosystem level the average of *k* values obtained incubating leaf litter in several habitats within the reach, but ignoring how decomposition varies within the reach. We suggest that only considering the heterogeneity of water velocity, values of *k* at habitat scale could be up-scaled at system level. Furthermore, we provide a novel method to standardize litter decomposition *k* by the influences of water velocity during decomposition process. In fact, we calculated *k* in velocity-days basis instead of day basis (chapter 5). This method could be a useful tool especially when other controlling factors of leaf litter decomposition are assessed.

Previous studies suggests that mineralization of leaf litter constituents by microbial assemblages can be maximized at stream locations with low water velocities, whereas physical fragmentation of leaf litter is higher at locations with fast water velocities (Ferreira et al. 2006). We also suggest that the positive relationship between water velocity and *k* observed in this thesis could be explained by leaf litter fragmentation. However, our results also suggest that the influence of water velocity on leaf litter decomposition can go beyond physical fragmentation and can also be explained by biological degradation. In fact, results from enzyme activity support this suggestion since the capacity of microbial decomposers to degrade cellulose polymeric compounds (i.e., CBH activity) was higher under low water velocity conditions, and it sharply decreased with increasing water velocity. Therefore, our results indicate that water

velocity not only influence on leaf litter export and the spatial distribution of leaf litter within the reach, but also in the main process accounting for leaf litter processing. On the one hand microbial mineralization, which can be maximized on leaf litter retained under low-velocity conditions. On the other hand, physical fragmentation which converts leaf litter into fine particles easily exported downstream. Therefore, we suggest that only the fraction of leaf litter inputs which are retained in relatively low-velocity locations can be entirely used by streambed communities. Therefore, even in low order streams the use of leaf litter has an efficiency, which may be dictated by the water velocity heterogeneity within the reach.

Considering all our results together, we found that water velocity is a factor controlling different aspects of leaf litter dynamics in streams because not only influences the fraction of leaf litter retained/exported but also how these inputs are spatially distributed and further processed within the streams. Therefore, water velocity should be considered in future studies to increase our understanding of how leaf litter inputs can effectively fuel the metabolism of stream ecosystems.

9.2. Influence of the riparian composition on in-stream solute dynamics

Composition of riparian tree species ultimately determines the quality of the leaf litter inputs and their further processing in streams (Webster and Benfield, 1986; Gasith and Resh, 1999; Ferreira et al. 2016). Thus, riparian composition can significantly influence on leaf litter dynamics, ecosystem metabolism and secondary production of receiving streams (Petersen and Cummins 1974, Gasith and Resh, 1999; Wymore et al. 2015). Inputs of high-quality litter such as alder (*Alnus glutinosa*), ash (*Fraxinus excelsior*) and black poplar (*Populus nigra*) are highly-bioavailable substrates for microbial and macroinvertebrate communities in streams. Thus, these species have an important ecological influence on the metabolism and the secondary production of forested headwater streams (Webster and Benfield, 1986; Woodward et al. 2012; Ferreira et al. 2014). In contrast, low-quality species such as black locust (*Robina pseudoacacia*) and sycamore (*Platanus X hispanica*) have been associated with low microbial activity, low rates of breakdown and decomposition (Gesner and Chauvet, 1994); and thus, they can have a low influence on the metabolism of recipient streams (Webster and Benfield, 1986). However, the present thesis suggest further influences of riparian composition on ecological status of streams and indicates that leaf litter quality also determines the interaction between leaf litter processing and the dynamics of solutes in the water column (Webster et al. 2009). In fact, we provide empirical evidences about how different leaf litter species (i.e., alder, ash, black poplar black locust and sycamore) may determine the bioavailability of leachates from leaf litter to streams, the microbial requirements of DIN and DOC from water column during decomposition process and how these demands are related to the activity of microbial decomposers. We also suggest that leaf litter quality can influence microbial-driven leaf litter decomposition among streams, which covered a wide gradient of inorganic nutrient concentrations.

The present thesis found that alder´s leaves provide the most bioavailable leachates to streams because these leaves release high amounts of dissolved organic nitrogen (DON) and NO_3 to streams (chapter 6; Table 9.1.). The dominance of the other species in the riparian zone may decrease ~3-4 times the microbial bioavailability of leachates in comparison to alder. Therefore, the presence of alder in the riparian zone could enhance the pool of dissolved organic matter and nutrients of the recipient streams. Nutrients provided by leachates from alder can affect microbial heterotrophic functioning either directly, by influencing nutrient uptake (Caron 1994), or indirectly, influencing the activity of primary producers (Romaní and Sabater 2000). Therefore, large amount of alder from riparian zones may stimulate overall stream activity during leaf litter senescence, especially in nutrient limited systems. Likewise, the plantation of alder in the riparian zones of nutrient poor systems may be an interesting tool to enhance and/or recover the ecological status of the stream, at least, during fall. In addition, we indicate that high-decomposing species such as alder and ash can strongly influence the strategies of microbial assemblages inhabiting on leaf litter to obtain matter and energy (i.e., trophic strategies). In fact, although microbial assemblages associated with alder and ash showed similar or even lower production of exoenzymatic activity of cellobiohydrolase (CBH) to that from the other species, they showed the lowest values of CBH turnover activity (TA) (chapter 7; Table 9.1.). This suggest that microbial assemblages associated to alder and ash efficiently rely on leaf litter tissues to sustain their enzymatic activity, which is in agreement with high decomposition rates found for these species (i.e., $k \sim 0.00163$ and $0.0103\ d^{-1}$, for ash and alder respectively; chapter 7, Table 9.1.). In the other species, microbial activity may be partially fueled by leaf litter tissues but also by external resources (i.e., from water column) (Romaní et al. 2006; Artigas et al. 2007). In fact, it has been observed that the different microbial groups

forming the stream benthic community show a wide range of trophic strategies to obtain energy. In general, algae and bacteria take nutrients from the water column by diffusion mechanisms, whereas heterotrophic bacteria and fungi can breakdown polymeric compounds and assimilate low-molecular weight compounds. Here, we suggest that different trophic strategies could even be showed among different leaf litter species; and that leaf litter quality may dictate the degree at which microbial assemblages rely from leaf litter and by the contrary, use leaf litter as a substrate and rely on elements from the water column. Our results of ^{15}N uptake during alder decomposition support this hypothesis revealing that microorganisms inhabiting in high-decomposing species (i.e., high-*k*) resulted to be less efficient in assimilating DIN from water column in comparison with the low-decomposing species (chapter 7). These results support the predictions from a stoichiometrically explicit computer model developed by Webster et al. (2009), which indicated that if nutrients are easily available from leaf litter they are taken up from the substrate first. These authors also hypothesized that on high-quality litter the uptake of nutrients from water column only occurs if needed. Interestingly, we also observed that microbial uptake of DIN during leaf litter decomposition was oppositely related to the uptake of DOC. Microbial assemblages inhabiting on high-decomposing species may be less dependent from the water column-N; but depend on DOC in a greater extent and viceversa. The high use of DOC in high-decomposing species could be explained by the rapid consumption of C-labile molecules in litter (i.e., high *k*). DOC could be used as labile-C resource. N uptake from water column during decomposition period varies ~ 43% (C.V.) among leaf litter species with the lowest and highest values for ash and sycamore, respectively (Table 9.1.). C uptake from water column varies ~64% among species which suggest that the quality of leaf litter species influences in a greater extent the uptake of DOC than the uptake of DIN. DOC showed

opposite patterns among species than DIN with sycamore and ash as the lowest and highest assimilating species, respectively. Sycamore can remain in the stream ~526 days (1/*k*) whereas ash may be decomposed in 98 days. Thus, sycamore not only can be a more efficient leaf litter assimilating DIN, but also can be a long-term colonizing substrate operating within the reach. In contrast, ash decomposes rapidly with higher demands of DOC, especially during later stages of decomposition process (chapter 7).

The present thesis suggests that black locust species is a remarkable exception comparing to the other species. Black locust is an allothonous species with high capacity to colonize riparian zones dominated by authocthonous riparian trees (…). The dominance of black locust in riparian areas could strongly influence the ecological status of the recipient streams. In fact, black locust is a N-fixing leaf litter species and thus it contains high N content relative to that C content (i.e., C:N ratio) (Table 9.1.). In concordance, we measured the highest values of the CBH accumulated enzyme activity (AEA) in black locust. However, leaf litter decomposition of black locust is relatively low suggesting that microbial enzyme activity is scarcely involved in leaf litter degradation. In concordance with this, values of turnover activity were the highest among species studied (Table 9.1.), showing that the microbial enzymatic activity on black locust was very inefficient decomposing leaf litter tissues (chapter 7). Our results corroborate previous studies which suggest that black locust should be considered as a low-quality litter regardless of its low C:N ratio. The low microbial efficiency and low decomposition found in black locust could be explained by the high proportion of recalcitrant compounds constituting the chemical structure of black locust such as lignin (Alonso et al., 2010) and the presence of polyphenols which forms complexes with proteins that are highly resistant to microbial activity and decomposition (Taylor et al.

1989; Hattenschwiler and Vitousek 2000). This suggest that the enzymatic activity in black locust may be sustained by organic compounds others than from leaf litter (i.e., from water column).

An essential prerequisite for the analysis of the ecosystem functioning is information on the biomass and activity of the main organisms constituting the system (Webster et al. 1990; Jone and Smock 1991). In forested headwater streams leaf litter inputs are the main energy source to streams and leaf litter decomposition has been stablished as the main tool to assess the ecosystem functioning and "in-stream services" within the systems (Cummins 1988; Woodward et al. 2012). To date, the protection and management of riparian vegetation and landscape focus on preserve streamside riparian vegetation (i.e., buffer zones) to prevent run-off and maintain a flux of leaf litter inputs to streams and forest floor (Meyer and Wallace 2001, Lowe and Likens 2005). However, this perspective overlook that leaf litter to streams is not only an organic matter source that decompose, but also a microbial colonizing substrate that interacts with the stream water column. The present thesis shed some light to the leaf-water column interaction and indicates that leaf litter quality strongly influence on C and N solute dynamics in streams. This interaction should be also considered to determine the importance of leaf litter as "ecosystem-function tool". Therefore, the managers of riparian zones and hence to the stream biodiversity should consider that leaf litter compartment (leaves + colonizing microbes) strongly interact with the stream-water column and that leaf litter quality control this interaction (chapter 6, chapter 7, chapter 8).

Table 9.1. Comparison of the different parameters examined as a function of the 5 leaf litter species studied. Where Rru is the Resorufin production, AEA-CBH is the accumulated enzymatic activity of CBH (cellobiohydrolase) and TA-CBH is the turnover activity of cellobiohydrolase.

In-stream processes	**Leaf litter Input**	**Leaf litter quality**	**Bioavailability of leachates**	**Leaf litter Decomposition**			**Solute uptake associated to leaf litter decomposition**	
Leaf litter species	Days of fall	C:N ratio	Rru production rates (mmol Rru g DM^{-1} min^{-1})	*k* (d^{-1})	AEA-CBH (mmols MUF gDM^{-1})	TA (mmols MUF gDM^{-1})	^{15}N-NH_4 Uptake rates (µg NH_4 mg N^{-1})	^{13}acetate Uptake rates (mg acetate g C^{-1})
Alder	166[a]	16.83[a]	5.19[a]	0.0103[b]	3.61	2	56.58	271.16
Ash	89[a]	17.32[a]	1.65[bc]	0.0163[a]	2.80	2.32	37.24	481.48
Black Poplar	85.5[a]	26.52[b]	2.25[b]	0.0080[b]	2.93	4	53.85	304.32
Black locust	80[a]	17.40[a]	1.56[bc]	0.0054[c]	5.51	12.25	76.32	125.41
Sycamore	----	46.77[c]	0.79[c]	0.0019[d]	1.33	7.9	111.69	76.09

CHAPTER 10: CONCLUSIONS

Chapter 5: spatial heterogeneity of water velocity drives the transport, spatial distribution and processing of leaf litter in streams

1. The retention of leaf litter in streams has commonly been assumed as a static process at base flow conditions; and only, a spatial re-distribution of retained leaves is associated with events of increasing discharge (i.e., floods). However, our results show that retained leaves within a reach can be spatially re-distributed over time and travel longer distances downstream even under stable discharge conditions.
2. Analyzing the spatial re-distribution of leaf litter throughout time, we found that leaves are effectively retained not only at sites with low water velocity velocities, but also under mid-to-fast velocity conditions (i.e., ~20 to 50 cm s^{-1}). Therefore, leaf litter inputs can be exposed to decomposition covering a wide gradient of water velocities.
3. We found a positive relationship between water velocity and leaf litter decomposition rates (*k)*, which is mainly explained by leaf litter physical fragmentation. Thus, water velocity becomes an important factor to be considered in leaf litter decomposition studies.
4. Results from this chapter demonstrate that leaf litter dynamics in streams is subjected to the hydro-morphological characteristics of the stream channels, since they determine the spatial heterogeneity of water velocity within the reach.

Chapter 6: Chemical and optical properties of different litter leachates influence in-stream nutrient pool and microbial activity

5. Leaf litter inputs from different riparian tree species produce leachates of different chemical and optical properties. The type of litter material entering into streams not only influences the quality of DOC but also the concentration of dissolved nitrogen (N) and phosphorous (P) of the leachates.
6. Microbial activity associated to leachates (estimated as Rru production rates) varies among leaf litter types. Dissolved organic N (DON) and NO_3 were the best predictors of differences on microbial activity among leaf litter types.
7. Leaf litter from Alder´s has the highest associated microbial activity comparing to the other species considered and thus, leachates from alder are sources of suitable dissolved organic matter and nutrients to streams.

Chapter 7: When leaf litter species matter, microbial uptake of ammonium and acetate from stream water during decomposition

8. The use of ^{15}N and ^{13}C stable isotopes revealed that decomposition stage of leaf litter and leaf litter species are important factors controlling the microbial demands of NH_4 and acetate from water column.
9. We found that microbial decomposers use N from water column since initial stages of the decomposition process, and that this N demand was relatively constant throughout the decomposition process. In contrast, microbial uptake of C-acetate from water column increased over decomposition time.
10. Among leaf litter species, the microbial uptake of NH_4 was negatively related to leaf litter *k* and positively related with the cellobiohydrolase use efficiency (i.e.,

TA), indicating that microbial assemblages rely in a greater extent of N from litter but also acquire N from water column.

11. Microbial uptake of acetate was positively related to leaf litter decomposition and to the accumulated cellobiohydrolase activity (i.e., AEA). Thus, microbial assemblages on high-decomposing leaf litter acquire C-acetate from the water column in a greater extent than that colonizing low-decomposing litter.

Chapter 8: Responses of microbially driven leaf litter decomposition to stream nutrients depend on litter quality

12. The response of microbially-driven leaf litter *k* to the stream nutrient gradient differed between leaf litter species of different quality. In particular, decomposition rates of high-quality litter such as alder decreased along the nutrient gradient, while no significant changes were observed in decomposition rates of low-quality litter such as sycamore species across the nutrient gradient.
13. Temporal variation of leaf litter N content across the nutrient gradient differed between alder and sycamore. These results indicate that, regardless of the stream conditions, leaf litter quality play a relevant factor controlling microbial colonization dynamics on leaves.
14. We found that cellobiohydrolase and phosphatase exoenzyme activities associated to alder and sycamore increased as DIN concentration in water column increased. However at DIN concentration >1mgN L^{-1} the two exoenzymatic activities are significantly depressed. These suggest that high DIN concentrations and/or high degree of pollution, typically observed in streams influenced by human activities, may deplete the production of enzymatic activities of microbial decomposers, and hence, leaf litter decomposition.

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ANNEXES

CHAPTER 5: Spatial heterogeneity of water velocity drives the transport, spatial distribution, and processing of leaf litter in streams

Table S5.1. Measurements of the water velocity distribution within the study reach where additions of leaves were conducted. Measurements were done at 23 transects along the 70 m-long reach. At each transect velocity was measured at 20 cm intervals. The range of measured water velocities (i.e., from 0 to 92 cm s^{-1}) was grouped at velocity intervals of 5 cm s^{-1}, resulting in a total of 18 categories. The relative proportion of locations within each water velocity category was calculated by dividing the number of locations for a given velocity category by the total number of locations measured within the reach (i.e., a total of 342 measurements).

Water velocity category	Velocity range (cm s^{-1})	Number of locations	Relative proportion
1	0-5	196	57.31
2	6-10	46	13.45
3	11-15	47	13.74
4	16-20	5	1.46
5	21-25	12	3.51
6	26-30	3	0.88
7	31-35	6	1.75
8	36-40	4	1.17
9	41-45	1	0.29
10	46-50	4	1.17
11	51-55	4	1.17
12	56-60	2	0.58
13	61-65	2	0.58
14	66-70	2	0.58
15	71-75	2	0.58
16	76-80	2	0.58
17	81-85	2	0.58
18	85-90	2	0.58

Table S5.2. Decomposition rates (*k*, with standard error of the regression in parenthesis) in units of both days^{-1} and velocity days^{-1}, the coefficient of determination (r^2) of the regressions and the number of leaf bags used during the exposure time. All regressions to estimate k from mass loss over time significant ($p < 0.001$). Different letters indicate significant differences in water velocities and leaf litter *k* among locations based on one-way ANOVA and one-way ANCOVA analysis, respectively, followed by post-Hoc Tukey´s t-test.

Sampling locations	Water velocity (cm s^{-1})	*k* (days^{-1})	r^2	Velocity standardized-*k* (velocity day^{-1}, s cm^{-1})	r^2	N
1	~0.00^{a} (0.00)	0.0076^{a} (0.0009)	0.77	___	___	24
2	0.07^{b} (0.03)	0.0081^{a} (0.0008)	0.79	0.00107 (0.000107) ^{a}	0.82	24
3	0.15^{c} (0.03)	0.0092^{a-b} (0.0008)	0.86	0.00058 (0.000045) $^{a-b}$	0.88	24
4	0.29^{d} (0.02)	0.0124^{a-b} (0.0008)	0.92	0.00043 (0.000025) ^{b}	0.93	23
5	0.50^{e} (0.02)	0.0148^{b} (0.0008)	0.93	0.00029 (0.000017) ^{b}	0.93	24
6	0.53^{e} (0.04)	0.0121^{a-b} (0.0008)	0.89	0.00023 (0.000016) ^{b}	0.90	24
7	0.78^{f} (0.03)	0.0205^{c} (0.0012)	0.89	0.00026 (0.000011) ^{b}	0.96	24
8	0.92^{f} (0.05)	0.0222^{c} (0.0009)	0.96	0.00024 (0.000017) ^{b}	0.90	23

Table S8.1. Longitudinal (Long.) and latitudinal (Lat.) location of the streams, average and SEM (in parenthesis, n=21) of physical and chemical variables for each stream during the study period, decomposition rates (*k*) for alder and sycamore and the ratio between decomposition rates of both alder and sycamore leaf litter. Different letters indicate significant differences on *k* based on one-way ANCOVA analysis and in the rest variables based on ANOVA analysis, followed by post-Hoc Tukey´s t-test. Note that for *k* capital and lower case letters indicate statistical differences among streams and between leaf litter species, respectively. DIN= dissolved inorganic nitrogen (nitrite + nitrate + ammonia). Streams influenced by wastewater treatment plant inputs are indicated with asterisks.

Parameters	DIN ($\mu g\ N\ L^{-1}$)		SRP ($\mu g\ P\ L^{-1}$)		DIN:SRP ($\mu g\ N\ \mu g\ P^{-1}$)	
Alder	Equation	r^2	Equation	r^2	Equation	r^2
Decomposition rate	0.0014 - 3.09e-7 DIN	0.77^{***}	0.0014 – 7.42e-6 SRP	0.93^{***}	ns	
Leaf litter N concentration	2.62 + 0.0002 DIN	0.66^{**}	2.61 + 0.004 SRP	0.34^{*}	ns	
Cbh activity	ns		Ns		ns	
Phos activity	ns		Ns		ns	
Sycamore						
Decomposition rate	ns		Ns		ns	
Leaf litter N concentration	1.85 + 0.0002 DIN	0.77^{*}	1.79 + 0.005 SRP	0.79^{***}	ns	
Cbh activity	ns		Ns		ns	
Phos activity	ns		Ns		ns	

CHAPTER 9: General discussion

Assessment leaf litter export across streams

During 2017, we performed a pilot study to investigate how leaf litter transport operates under baseflow conditions across streams of different hydro-morphology characteristics and how the heterogeneity of water velocity would explain the leaf litter export among streams. To tackle this objective, we conducted *Ginkgo biloba* additions on 6 stream reaches (~80m length) which covered a wide gradient of stream discharge and streambed morphology. To conduct the additions of leaves we followed the methodological approach mentioned in the present thesis (chapter 5). The additions of leaves were conducted in reaches of La Tordera catchment where most of our experiments were conducted. The time frame between the addition and collection of the Ginkgo leaves was ~3 days in order to account with the spatial re-suspension of the leaves within the channel (chapter 5). We related the percentage of leaves exported downstream with the stream discharge and also with the heterogeneity of water velocity found within the reach. For this pilot study, the heterogeneity of water velocity of each study reach was calculated based on cross section transects, where water velocity was measured each 20 cm wide every 3 m long covering the total reach length. In each study reach, the range of water velocities measured was grouped at velocity intervals of 5 cm s^{-1} (i.e., velocity categories). The relative proportion of water velocity measurements of each category was calculated by dividing the number of locations of each category by the total number of locations measured within the reach (Table S9.1).We determined the heterogeneity of water velocity by calculating the Coefficient of Variation (CV) among the relative proportion of each velocity category (Table S9.1.).

Table S9.1. Data from the additions of Ginkgo biloba leaves conducted in 6 streams reaches within La Tordera catchment. We Show the number of measurements of water velocity within the reach, the recovery factor for each leave addition, the percentage of leaves exported from the study reach, the stream discharge on each study reach and the heterogeneity of water velocity expressed as CV values among each water velocity category (see more details in *assessment leaf litter export across streams;* Annexes; General discussion section).

Stream order	Number of velocity measurements (units)	Recovery factor (% from added leaves)	Leave export (% from added leaves)	Stream discharge (L s^{-1})	Heterogeneity of water velocity (CV among water velocity categories)
1	342	100	0	20.58	2.44
2	279	97	54.5	82.96	1.62
3	398	96	88	951.71	0.65
1	313	88	49.5	28.97	1.57
2	575	90	73.5	126.32	1.58
2	348	94	83	86.70	0.83