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Leaf litter decomposition in a fluvial-estuarine environment

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Professora Doutora Cristina Maria Moreira Monteiro Leal Canhoto (Universidade de Coimbra)

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Para a minha família...

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RESUMO

Ainda não se conhece a importância das folhas nas áreas fluvio-estuarinas enquanto fontes de nutrientes para as cadeias alimentares aquáticas. Neste estudo avaliámos a decomposição da folhada de amieiro (*Alnus glutinosa*) e da de choupo (*Populus nigra*), bem como as comunidades microbianas e a meio- e macrofauna na zona fluvio-estuarina de transição do Rio Mondego. As folhas foram expostas por 21 dias em sacos de malha grossa e fina, estando sujeitas a oscilações de salinidade na água durante o período de condicionamento.

Não foram encontradas diferenças nas taxas de decomposição entre folhas, todavia o choupo demonstra uma tendência para ter valores mais altos de k (dia^{-1}). A respiração microbiana foi significativamente mais alta nas folhas condicionadas nos sacos de malha grossa (MG), possivelmente devido a uma maior oxigenação do substrato. A biomassa fúngica foi vestigial nas folhas de amieiro e de choupo condicionadas nos sacos MG. Verificou-se uma baixa riqueza fúngica e as taxas de esporulação não diferiram entre espécies de folha; no entanto, os fungos que colonizaram o choupo produziram > 3,09 vezes mais esporos que os do amieiro. Observou-se que a abundância bacteriana foi significativamente mais alta nas folhas de choupo ($p > 0,05$), o que sugere a importância deste grupo procariótico na degradação de folhas mais recalcitrantes. A abundância de meiofauna tendeu a seguir a abundância bacteriana, mas não se verificaram diferenças estatísticas entre espécies de folhas ($p > 0,05$); os macroinvertebrados foram quase inexistentes.

A contribuição dos fungos e macroinvertebrados na decomposição foliar parece ser negligenciável nestas zonas de transição. A decomposição da folhada e o ciclo de nutrientes nas margens da área fluvio-estuarina parece ser maioritariamente promovida por bactérias e por uma comunidade de meiofauna resistente dominada por nemátodes.

Ciclos de imersão e desidratação nestas zonas podem ter um papel chave para determinar os protagonistas do processo de degradação das folhas. Estudos adicionais são necessários para entender o destino e importância relativa da folhada nestes sistemas altamente variáveis.

Palavras-chave: fluvio-estuarina, folhada, decomposição, bactérias, meiofauna, factores abióticos.

ABSTRACT

The importance of leaves in fluvial-estuarine areas as source of nutrients to the aquatic food webs is still not known. In this study we evaluated the decomposition of alder (*Alnus glutinosa*) and poplar (*Populus nigra*) leaf litter and associated microbial, meio- and macrofauna in the fluvial-estuarine transitional area of the Mondego River. Leaves were exposed in coarse and fine mesh bags for 21 days, subjected to oscillations in water salinity during the conditioning period.

No differences were found in decomposition rates between leaves, however poplar shows a tendency to have higher values of k (day^{-1}). Microbial respiration was significantly higher in leaves conditioned in coarse mesh bags, possibly due to better aeration of the substrata. Fungal biomass was vestigial in alder and poplar conditioned in CM bags. Fungal richness was low and sporulation rates did not differ between leaf species; however, fungal colonizing poplar produced >3.09 times more spores than alder. Bacterial abundance was significantly higher in poplar leaves ($p< 0.05$), suggesting the importance of this prokaryotic group in the degradation of the more recalcitrant leaves. Meiofauna abundance tended to follow bacterial abundance but no statistical differences were observed between leaf species ($p> 0.05$), and the macroinvertebrates were almost inexistent.

The contribution of fungi and macroinvertebrates in leaf decomposition seems negligible in these transitional salt richer areas. Litter decomposition and nutrient cycling in the margins of the fluvial-estuarine area appears to be mainly promoted by bacteria and by a resistant meiofaunal community dominated by nematodes. Cycles of submersion and dehydration in these areas may play a key role determining the protagonists of the breakdown process in these areas. Further work is still needed to

understand the fate and relative importance of leaf litter in these highly variable systems.

Key-words: fluvio-estuarine, leaf litter, decomposition, bacteria, meiofauna, abiotic factors.

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- CHAPTER 1 -

General Introduction

1.1 The River Continuum Concept (RCC)

The River Continuum Concept (RCC) is a central tenet in stream ecology proposed by Vannote *et al.* in 1980. This theory states that the stream is a gradient of physical conditions (e.g. width, depth, flow volume) which affects the structure and function of communities along its system; this is largely regulated by fluvial geomorphic processes and environmental factors (Vannote et al., 1980; Magdych, 1984; Grubaugh et al., 1997; Tomanova et al., 2007). It was based in studies performed in a pristine stream system in North America and, initially consisted of five propositions; four of them were later on argued by Statzner & Higler (1985), remaining generally accepted the first principle which stated that river systems featured a continuum, whose physical conditions gradually change down the stream, controlling the composition of the aquatic community; the links between up and downstream are also enlightened as Vannote and co-workers also stated that downstream communities benefit from the *wastes* of the upstream processing – this holistic point of view of the river system was verified in studies performed in temperate streams and rivers (Montgomery, 1999; Jiang et al., 2011). The RCC is until now a popular concept in community researches (Rosenfeld et al., 2007; Webster , 2007; Jiang et al., 2011).

In temperate forested streams, the riparian vegetation provides, not only, shade to the water – especially in low order streams – but also an allochthonous input mainly composed of leaves and stems that fall into the stream (course particulate organic matter – CPOM) (Vannote et al., 1980; Grubaugh et al., 1997;

Gessner et al., 1999 & 2010). As the stream order increases, the river widens and the importance of the CPOM input decreases; the system becomes less retentive for organic matter (Richardson & Danehy, 2007). However, the more wide the streams get, the less shading it receives, increasing the autochthonous production. These differences in production result in a U-shaped curve of energy input (Grubaugh et al., 1997; Webster, 2007). This is also influenced by the spiraling of nutrients which explains how the longitudinal transport in streams works. The term "nutrient spiraling" elucidates how the flow of nutrients works from upstream to downstream: the energy ingested by one organism is later egested and returns to the pool of energy or detritus, to be used again downstream (Webster & Patten, 1979).

Therefore, the functional and structural characteristics of the communities adjust themselves to this gradient, while being conditioned by the type of organic matter in the stream – the bigger the stream, the lower importance of the riparian area. Along the path from head to mouth of the river, the fragmentation effects, resulting of physical and chemical processes, slowly converts the CPOM (coarse particulate organic matter, ⌀ > 1mm) into DOM (dissolved particulate organic matter, ⌀ < 0,45µm) and FPOM (fine particulate organic matter, 0,45µm < ⌀ < 1mm), which is abundant near the estuary. In another words, the stream characteristics affect the type and availability of food resources, which has different outcomes in the communities along the continuum.

The communities of invertebrates on the continuum are distinguished not by what they eat, but by the way they capture their food, being divided in functional feeding groups: shredders, collectors, scrapers and predators (Fig.1) (Cummins & Klug, 1979; Vannote et al., 1980; Richardson & Danehy, 2007; Tomanova et al., 2007).

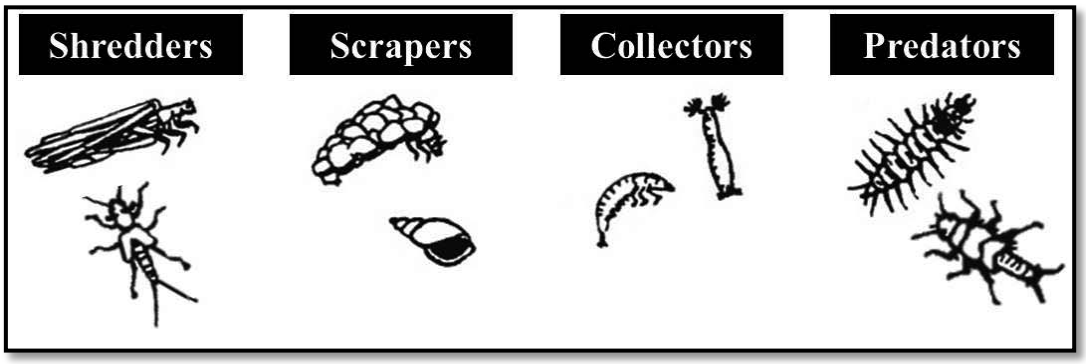

Fig. 1: Illustration of some stream invertebrates. Adapted from Allan & Castillo (2007).

In shallow covered streams, more easily found where the flow of the river starts, there is abundance in shredders and collectors, who feed on large particles of organic matter (CPOM) by shredding them, and fine particles of organic matter (FPOM) by filtering the matter from the water or by gathering the particles, respectively (Cummins et al., 1973; Cummins & Klug, 1979) (Fig.2).

Studies like the one from Greenwood (2007) relate the abundance of shredders with the fungal biomass, since the macroinvertebrates prefer to consume fungi conditioned leaf material. The ratios of production/respiration (P/R) are, usually, below 1, revealing a heterotrophic system (Vannote et al., 1980; Grubaugh et al., 1996 & 1997).

Downwards on the stream, where the light is able to shed on the channel, there is an occurrence of algae and aquatic macrophytes, which increase the primary production, being the base of the trophic chain in medium order streams. The importance of shredders diminishes, since there's less CPOM accessible, and the number of grazers increases, since they feed on periphyton by rasping the surface where it is attached (Cummins & Klug, 1979). Now, the P/R ratios are higher than 1, since there's a high level of photosynthesis, and the environmental heterogeneity is maximum (Vannote et al., 1980; Grubaugh et al., 1997; Beketov & Liess, 2008).

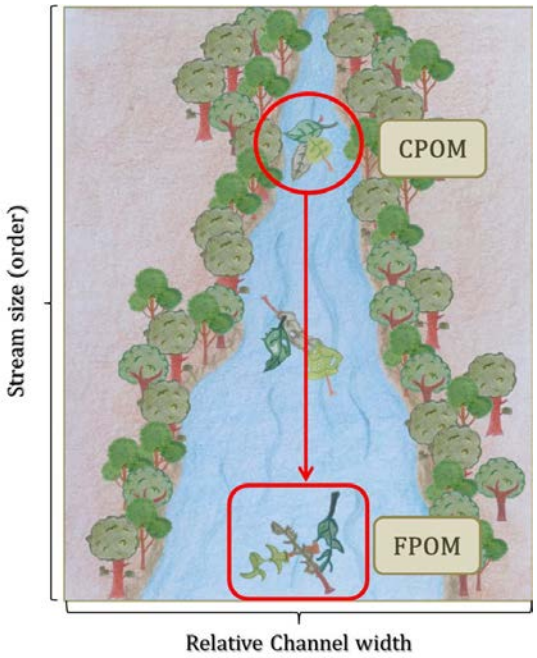

Fig. 2: Illustration of the River Continuum Concept and the flow of organic matter.

In the lower reaches, collectors dominate, as light penetration and aquatic photosynthesis occurs only in the upper part of the water column. Furthermore, the substratum here is composed of gravel and silt, not allowing the growth of periphyton. At this point, the system becomes heterotrophic, with its main source of energy being the FPOM from upstream (Fig.2) (Vannote et al., 1980; Grubaugh et al., 1997). The P/R ratios are lower than 1 again. Predators, who prey on other animals (Cummins & Klug, 1979), are a little distributed all along the stream (Vannote et al., 1980).

1.2 Fluvial-estuarine environments

Headwater systems are highly dependent on organic matter from allochthonous sources (like leaves) as an input of energy. The importance of this

input of organic matter derived from the terrestrial environment in large rivers, particularly in the fluvial-estuarine area, is still unknown but likely more important than expected (Fuentes-Cid et al., 2014). Leaves may appear in this zone as an input from the riparian area – either by falling directly onto the estuary or by plain flooding – or from upstream transport, especially in months when the precipitation is more abundant, increasing the flow of water.

The fluvial-estuarine environment is a transitional area where we find strong gradients in variables such as salinity (ranging from that usual of freshwater to those typically marine) (Nogales et al., 2010). Little is known of the protagonists of the incorporation into secondary production of this leaf material in these dynamic areas. Some studies (e.g. Lecerf, 2008), suggest that bacteria might have a significant role in linking the organic matter through the food-web while it was found that the richness in freshwater invertebrates decreases with the increase of salinity (Piscart et al., 2006).

Leaf litter decomposition

Leaf litter decomposition occurs in three more or less overlapping phases: leaching, conditioning and physical and biological fragmentation. The first abiotic phase consists in loss of a large amount of soluble leaf compounds (up to 30%), which occurs relatively fast (24 hours) (Gessner et al., 1999). Conditioning corresponds to the colonization of leaf by microorganisms mainly fungi, bacteria but also by a frequently neglected meiofauna (Robertson et al., 2000). Fungal colonization enhances the nutritional quality of dead organic matter for the invertebrates, turning the leaf into a more palatable and nutritious substratum for its consumers. Microorganisms, mainly fungi, are known to improve leaf litter quality

due to enzymatic and mechanical activity that modifies the leaf matrix, softening the tissue and increasing its nutritive value (Gessner et al., 1999; Chung & Suberkropp, 2009; Bärlocher, 2010; Gessner et al., 2010). Meiofauna (or meiobenthos) are small benthic animals that by definition pass through a 500µm sieve, but are retained on meshes of 40 – 64µm (Coull, 1999). These invertebrates are functionally important in estuaries for many reasons: (i) stimulate bacterial growth that consequently facilitates and enhances the mineralization of organic material and nutrient regeneration, (ii) contributes as food for many higher trophic levels, (iii) are highly sensitive to anthropogenic modifications, helping to better understand the influence of pollution (Coull, 1999; Hourston et al., 2011).

Leaf litter decomposition in fluvial-estuarine environments: sparse evidences

It is hypothesized that the input of allochthonous organic matter (CPOM) may still be significant in large rivers (high order). Regarding the microbial communities in these rivers, the fungi seem to dominate while the leaves are intact, whilst the bacteria abundance rises proportionally to the increasing fragmentation of the CPOM (Baldy et al., 1995). In other words, fungi are more present while the leaf is intact and the bacteria only increases after some breakdown of the leaf. Studies show a complementation between fungi and bacteria and not a replacement: bacterial biomass rises with the decrease of the fungal biomass; they're both present at the same time. This supports the idea that the greater the breakdown is, so it will be the participation of bacteria (Baldy et al., 1995). This phenomenon also seems to occur in salt influenced areas like salt marshes (Buchan et al., 2003).

Aquatic hyphomycetes, particularly some species, are able to grow (and eventually sporulate) and promote leaf decomposition under laboratory conditions, at high NaCl concentrations (Simões et al., submitted), which make them, along other fungal groups (Fig. 3) and bacteria (Baldy et al., 1995), candidates to mediators of leaf degradation in these transitional waters.

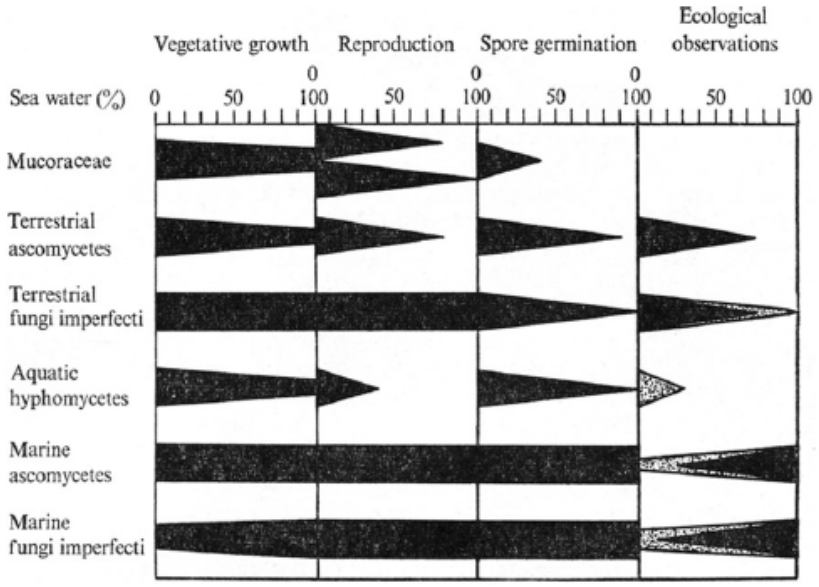

Fig. 3: Graph adapted from Byrne & Jones (1975), summarizing salinity tolerances of groups of fungi.

Sridhar & Bärlocher (1997) using pure salts at levels that mimic the transition from fresh to brackish to salt water, indicate a reduction or total inhibition of sporulation of these fungi at 22% and 44%, respectivelly.

Roache et al. (2006) tested the influence of different concentrations of salt on the degradation of leaves of *Triglochin procerum*. They observed that high values of NaCl inhibit the microbial enzymatic activity on leaves, leading to less effective leaf decay.

In fluvial-estuarine transitional waters abiotic factors such as tides, turbidity, oxygen concentration and, obviously salinity, are import determinants of the aquatic communities and processes performed by the ecosystem. The few studies focusing leaf litter decomposition indicate a small influence by the invertebrate decomposers (Fuentes-Cid et al., 2014), a crucial importance of bacteria (Crump et al., 1998 & 1999) and a possible participation of fungi (Simões et al., submitted) in the process. Indeed, several studies (Jones, 2000; Tsui & Hyde, 2004; Van Ryckegem & Verbeken, 2005) confirmed the presence of fungi in these environments although diversity losses and changes in the species composition of fungal communities may be expected along the fresh-salt water gradients potentially affecting decomposition (Van Ryckegem & Verbeken, 2005).

The experiences performed with *Phragmites* sp. (Tanaka, 1991; Van Ryckegem & Verbeken, 2005; Lopes et al., 2011) and other reed and algae litter, tend to agree on an inverse relationship between the water salinity and litter breakdown.

1.3 Objectives

In this study we assess the decomposition of *Alnus glutinosa* and *Populus nigra* leaf litter and its colonization by microorganisms, meiofauna and macroinvertebrates in a fluvial-estuarine area of the Mondego River . We expect the decomposition to be faster in less recalcitrant leaves as alder. We anticipate that, due to salinity effects and to an impoverished leaf-consuming invertebrate community, the decomposers, mainly bacteria, and meiofauna will have a predominant role in the leaf decomposition process.

- CHAPTER 2 -

Material and Methods

2.1 General

Estuary characteristics and water parameters

The Mondego basin has an area of 6670 km^2 alongside the western coast of Portugal (40º08'N, 8º50'W). The river's estuary is 21 km long and is influenced by a warm-temperate climate (Alves et al., 2013).

Regarding our experimental site, we tried to assess an area of transition, where we see the clash of fresh and salt waters, with the influence of both river and tides. The range of salinity values previously registered for the chosen area were 18-30 (Teixeira et al., 2008) from the year of 2002 until 2005. Values registered during the study period (October 2013) were 15.90 ± 0.87 (mean ± SD).

In situ water parameters were evaluated using YSI Professional Plus Multiparameter instrument: temperature, salinity, conductivity, pH and oxygen (O_2). Water samples were retrieved and then transported to the laboratory, for posterior chemical analysis in a Skalar San++ system Multiparametric Autoanalyser: NH_4 (ammonia), Si (silica), NO_2 (nitrate), NO_3 (nitrite) and PO_4 (phosphate).

Initial litter quality

Alder (*Alnus glutinosa*) and poplar (*Populus nigra*) leaves were collected after their senescence in Coimbra's Parque Verde (N:40º12'5, W:8º25'30) and Choupal (N: 40º73'17, W:8º27'20), respectively, air dried in the dark at room

temperature and stored until need. These species were chosen since they are common on the edges of Mondego River in the fluvio-estuarine zone. The initial and final (after 21 days incubation) phosphorus (P; SRP method) (Eaton et al., 1995), carbon (C), nitrogen (N; IRMS Thermo Delta V advantage with a Flash EA 1112 series) and total phenolic concentrations (Bärlocher & Graça, 2005) were determined for both leaf species, to allow the characterization of initial leaf quality. Initial toughness (n=3) was also evaluated according to Graça et al. (2005).

Litter bags and decomposition

For our experiment we used two types of mesh bags (10 x 15 cm) to enclose the leaves (after they were rehydrated): fine (FM – 0.5mm mesh diameter) and coarse mesh (CM – 1mm mesh diameter) bags. FM bags allow us to access the decomposition of the leaves based on the activity of the microbial community (bacteria and fungi) and meiofauna, while the CM bags also allow leaf processing by invertebrates. Each bag was labeled and contained a total of 2.01g ± 0.003 for alder, and 2.50g ± 0.002 (mean ± SD) for poplar. Bags were tied with nylon cords to the margins and were forced to immerse by the use of stones that kept them over the sediment. A total of 32 bags (16 CM and 16 FM) per species were introduced on October 2nd 2013 in the experimental site (Fig.4) and were recovered after 21 days of exposure. One extra group of each litter treatment (n=3) was taken to the stream on day 0, and brought back to the laboratory to estimate initial litter ash-free dry mass (AFDM) taking into account mass loss due to handling. Each bag was enclosed in zip-lock bags, and brought to the laboratory in a cooler in each sampling date. Once in the lab, leaves from each bag were gently rinsed with distilled water to remove attached sediments. From each coarse mesh bag, 5 sets of

3 leaf discs were cut with a cork borer (12mm diameter). One set of leaf discs was frozen in zip-lock bags at -20ºC for posterior assessment of ergosterol (proxy of fungal biomass); another set was used to assess sporulation rates. Two groups were preserved at -80ºC to preform DAPI (assessing bacterial numbers) or immediately used for microbial respiration (O_2 consumption) evaluation. The 5^{th} set was grounded and used for chemical analysis as indicated above. In the case of fine mesh bags, leaves were also used to evaluate mass loss and microbial respiration. Leaves were oven-dried for 48h at 105ºC and re-weighted for dry mass (DM) determination. Leaves were then combusted at 550ºC for 6h, and reweighed to assess ash-free dry mass remaining (AFDM). This procedure avoided overweighing due to inorganic sediment accumulation.

Fig. 4: Mondego estuary, October 2013 (photo: Ana Lírio).

2.2 Microbial parameters

Microbial respiration

Microbial oxygen consumption was determined after 21 days from a set of 3 leaf discs obtained from all bags (16 CM bags and 16 FM bags). Set of 3 discs were

immersed in water (25ml, dark flasks) from the study site saturated with oxygen at 19ºC.

The flasks were closed and left overnight. Oxygen evaluation was made with an oximeter (Oxi 3210, WTW, Weilheim, Germany) and oxygen consumption was expressed as mg O_2 g^{-1} AFDM h^{-1}.

Ergosterol

Ergosterol was used to estimate the fungal biomass associated with the leaves (Gessner & Chauvet, 1993; Graça et al., 2005).

The discs of each sample date preserved at -80ºC were lyophilized, weighted and put in the correspondent extraction flasks with the addition of methanol (CH_3OH) and sodium hydroxide (NaOH).

The extraction flasks were placed inside plastic bottles and heated in a microwave (400W; 20secs, 3 times with 3 minutes interval). The bottles are left to cool down for 15 minutes. The solution was then neutralized with hydrochloric acid (HCl) to facilitate the extraction.

Pentane (C_5H_{12}) and CH_3OH were added to the flasks to enable the separation of the ergosterol with the help of the vortex. When the two fractions were easily distinguished, the upper one – that consists of C_5H_{12} and ergosterol – was removed with a Pasteur pipette and transferred to a centrifuge tube. This extraction is done two more times, to waste the least possible.

We then proceed to the evaporation of the C_5H_{12} through the use of a sand bath at 55-60ºC inside the hotte. The centrifuge tubes were washed with pentane and transferred to the HPLC vials (done three times so that each vial contains ≈ 1,5 mL of C_5H_{12} and ergosterol). The pentane was evaporated from the vials again.

The sample was dissolved with methanol and shaken. Samples were kept at 4ºC until being read in the HPLC (High Performance/Pressure Liquid Chromatography). Ergosterol was converted to fungal biomass with a conversion factor of 5.5 µg ergosterol/mg fungal dry mass (Gessner & Chauvet, 1993). Fungal biomass was expressed as mg fungal biomass g^{-1} AFDM.

Sporulation rates

The 2nd set of 3 leaf discs (12 CM bags) was used to induce sporulation of aquatic hyphomycetes. Leaf discs were incubated in 100ml Erlenmeyer with 25 ml of filtered stream water and placed in an orbital shaker (100 rpm; 48 hours) at 16ºC. Each Erlenmeyer's suspension was transferred to a Falcon tube with 2 ml of formalin (37%). The volume was adjusted to 35 ml with distilled water. Counting and identification (Gulis et al., 2006) was done after filtering the conidial suspensions (Millipore SMWP, 5 µm pore size) and by staining the spores with 0.05% cotton blue lactic acid (60%) (Graça et al., 2005). The discs used in sporulation were dried (105ºC; 48 hours), weighed, ashed at 550ºC for 6 hours and reweighed. Sporulation rates were expressed as no. conidia / mg AFDMr /d.

Bacterial abundance

Bacterial abundance associated with leaf litter was estimated via direct counts using an epifluorescence microscope (Nikon Optiphot) after 4-6-diamidino-2-phenylindole hydrochloride (DAPI, Sigma) staining (Porter & Feig, 1980). Leaf discs were sonicated in a sonication bath (Selecta) to promote bacteria detachment. After dilution (10 times), the samples were stained with DAPI and collected in black 0.2 µm polycarbonated filters (Nucleopore, Whatman). Bacterial slide

counting was carried out at x1,000 magnification with a Nikon E600 epifluorescence microscope. At least 20 random fields were counted for each slide (Porter & Feig, 1980). Procaryotic cell abundance was expressed as no. individuals $^10^8$/ g AFDM.

2.3 Invertebrates

Meiofauna and macroinvertebrates

Leaves from CM bags were gently rinsed with distilled water into a 500 µm mesh sieve to retain attached invertebrates. The water was saved for further collection of meiofauna using a 38 µm mesh sieve (see below).

The invertebrates, were recovered, stored in scintillation vials, and preserved with 95% ethanol. Invertebrates were then sorted and identified under a binocular microscope (50x; Leica M80, Singapore). Identification was carried to the lowest taxonomic level possible following Hayward, et al (1995). After identification, the invertebrates were included in the correspondent functional feeding group (Hieber & Gessner, 2002).

Meiofauna – measured in number of individuals per gram of AFDMr – from the 12 CM bags that were trapped in the 38 µm mesh sieve were relocated into flasks with 37% formaldehyde for preservation and posterior counting and identification.

2.4 Statistical analysis

The chemical composition of alder and poplar leaves was compared using a two-way ANOVA (leaf species and incubation time as categorical variables). A t-

test was used to evaluate initial toughness of the leaves. The significance level was set at p=0.05.

We estimated decomposition rates (*k*) by linear regression of mass (ln transformed) over time, assuming a negative exponential model $M_t = M_O \times e^{-k}$, where M_t is the remaining mass at time t, M_o is the initial mass and *k* is the decomposition rate. *k* values were compared by a two-way ANOVA using both litter and mesh type as categorical variables, followed by Tuckey's test (Zar, 1999).

Microbial respiration of conditioned leaves was also assessed by a two-way ANOVA with leaf species and mesh type as categorical variables.

Sporulation rates, bacterial and meiofauna abundance were compared by one-way ANOVA, with leaf species as categorical variable in all cases. Tuckey's tests were applied whenever relevant. Data were transformed (log (x+1)) when necessary to satisfy assumptions of normality and homoscedasticity.

All statistical analyses were performed with STATISTICA 7.0 software.

- CHAPTER 3 -

Results

3.1 Litter quality and decomposition

The physic-chemical parameters of the water measured indicated a neutral pH and a high conductivity, likely due to salinity (Table I).

Table I: Physic-chemical parameters of the water in our study site. The values correspond to the mean ± SD; n=3.

Parameters	***Study site***
Temperature (ºC)	18.95 ± 0.04
Salinity	15.90 ± 0.87
Conductivity (mS/cm)	22.99 ± 1.10
pH	7.40 ± 0.803
Velocity (m/s)	0.66 ± 0.07
Oxygen (mg/L)	5.82 ± 0.05
Ammonia (ng/L)	62.83 ± 10.31
Nitrate (ng/L)	189.44 ± 49.41
Nitrite (ng/L)	118.87 ± 2,62
Phosphate (ng/L)	107.68 ± 8.89
Silica (ng/L)	1936.01 ± 342.68

The chemical composition of the leaves was measured in the beginning of the experiment and after 21 days of incubation in our experimental site (Table II).

Table II: Chemical composition of alder and poplar leaves before and after incubation (21 days; n=3). Results were expressed as percentage of ash-free dry mass remaining (AFDMr). The values correspond to the mean ± SD. Different letters indicate differences between treatments (2-way ANOVA: $p<0.05$).

		% C	% N	% P	% Phenols
Alder	**Before**	50.91 [a] ± 1.50	3.75 [a] ± 0.06	54.84 [a] ± 1.14	9.09 [a] ± 0.05
	After	38.95 [b] ± 0.86	4.15 [b] ± 0.07	42.47 [b] ± 1.85	6.19 [b] ± 0.00
Poplar	**Before**	42.98 [a] ± 2.96	1.88 [a] ± 0.04	64.61 [c] ± 1.65	12.95 [c] ±0.02
	After	35.85 [c] ± 0.46	1.18 [c] ± 0.12	33.07 [d] ± 5.87	6.24 [d] ± 0.00

Poplar leaves were statistically tougher (62.80 g ± 1.90) and, therefore, more recalcitrant than alder leaves (46.85 g ± 1.14) (mean ± SD) (t-test: $p=0.01$). Leaves degradation after 21 days immersion did not allow an accurate measurement of this parameter. However, parallel tests indicate that, after 7 days of immersion in our study site poplar leaves leachate >1.5 times more than alder leaves (personal observation).

Poplar lost more mass when in the fine mesh bags (FM) (38.23% ± 2.17 over 34.56% ± 2.01, in poplar and alder, respectively; mean ± SD), while alder lost more mass (36.85% ± 1.70) than poplar (33.91% ± 1.11) when incubated in coarse mesh bags (CM; mean ± SD).

No significant differences were found in decomposition rates (*k*) between treatments (2-way ANOVA: $F_{(3,20)}$=0.04; p=0.85. However, the more recalcitrant leaf (poplar) shows a tendency to have higher decomposition rates (Table III).

Table III: Decomposition rates (*k* day^{-1}) of alder and poplar leaves incubated in FM and CM bags for 21 days. Values are means ± SD; n=3.

Leaf type	Mesh	*k*(day^{-1})
Alder	**FM**	0.06 ± 0.01
	CM	0.07 ± 0.01
Poplar	**FM**	0.10 ± 0.02
	CM	0.09 ± 0.01

3.2 Microbial parameters

No statistical differences were observed in oxygen consumption between alder and poplar (2-way ANOVA: $F_{(3,20)}$=0.01; p=0.94). Mesh types showed statistical differences (2-way ANOVA: $F_{(3,20)}$=25.16; p= 0.00), being the oxygen consumption in coarse mesh bags of both leaves significantly higher than the consumption verified for fine mesh bags. The values of consumed oxygen registered were 2.78 mg O_2/ g AFDMr/ h ± 0.42 for the FM and 4.73 mg O_2/ g AFDMr/ h ± 0.36 (mean ± SD) in leaves incubated in CM (Fig. 5).

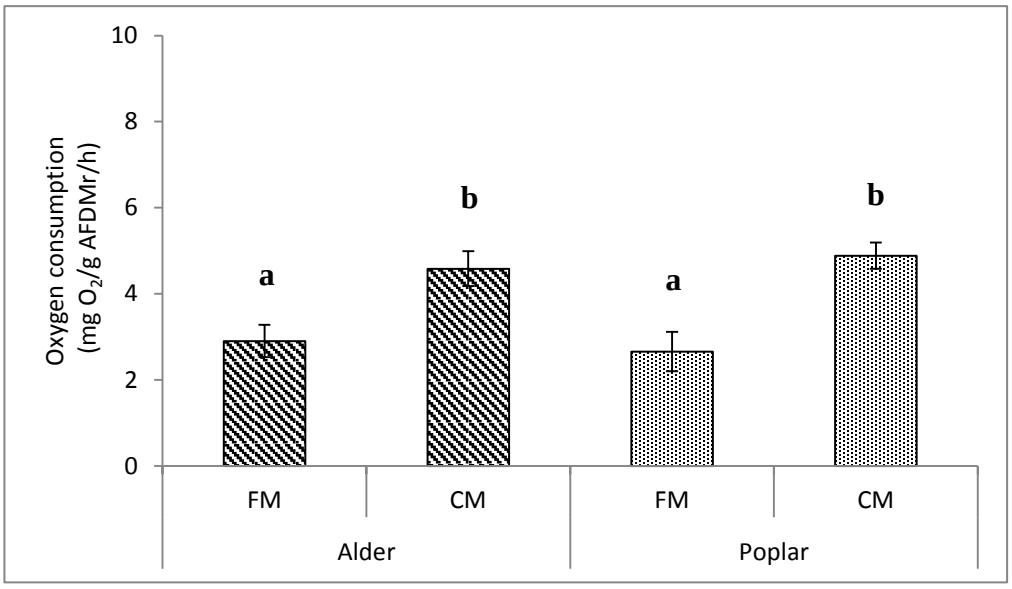

Fig. 5: Oxygen consumption (mg O_2/ g AFDMr/ h) by microbial communities associated with alder and poplar conditioned for 21 days in coarse (CM) and fine (FM) mesh bags. Values are means ± SD. Different letters indicate differences between treatments (2-way ANOVA, $p<0.05$; n=3).

Vestigial (< 2 mg g^{-1} AFDMr) values of ergosterol were found in both leaf species.

Sporulation rates (Fig. 6) were low in both species: 5.44 ± 2.01 (alder) vs 16.84 ± 8.77 (poplar) no.conidia/ mg AFDMr/ d (mean ± SD). No statistical differences were found in sporulation rates between the two leaf species (one-way ANOVA: $F_{(1,10)}=1.61$; $p= 0.23$) after 21 days of exposure. In both leaves the majority of spores produced belonged to *Fusarium* sp. (> 75% in alder; > 80% in poplar); *Heliscus lugdunensis* and *Tetrachaetum elegans* were also found in alder and poplar, respectively (Fig. 7).

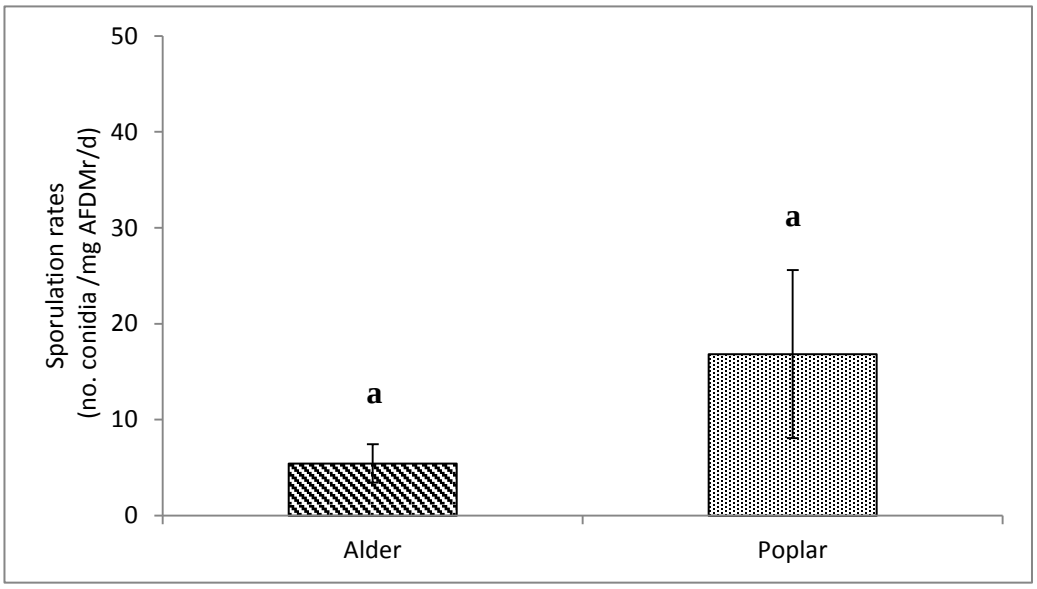

Fig. 6: Sporulation rates (no. conidia/ mg AFDMr/ d) found in alder and poplar after 21 days immersion at our experimental site. Values are means ± SD. Different letters indicate differences between treatments (one-way ANOVA, $p<0.05$; n=3).

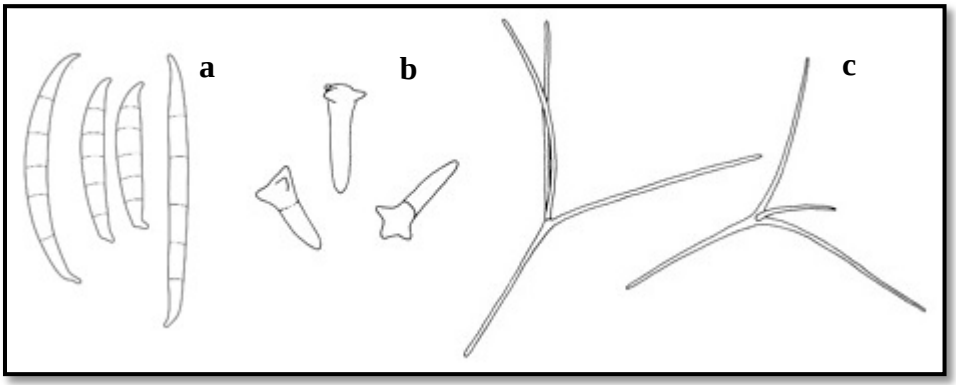

Fig. 7: Illustration of the spores found in alder (a, b) and poplar (a, c) after 21 days of incubation in our study site: a) *Fusarium* sp. (adapted from Yli-Mattila et al., 2009); b) *Heliscus lugdunensis* (adapted from Dang et al., 2007); c) *Tetrachaetum elegans* (adapted from Dang et al., 2007).

Statistical differences were registered in bacterial numbers between leaf species (one-way ANOVA: $F_{(1,10)}$=0.88; p= 0.02) after 21 days of exposure (Fig. 8). The values recorded were 1.56 × 10^8 ± 0.91 for alder and 6.95 × 10^8 ± 0.57 no. individuals/ g AFDMr (mean ± SD) for poplar.

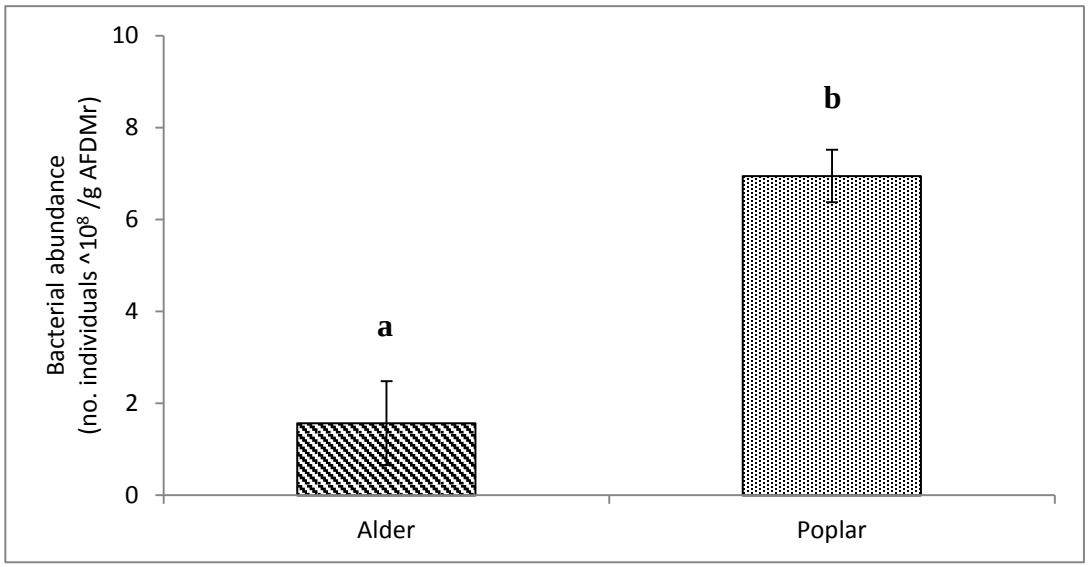

Fig. 8: Bacterial abundance (no. individuals^10^8/ g AFDMr) found in alder and poplar after 21 days of exposure at our experimental site. The values correspond to the logarithmic mean ± SD. Different letters indicate differences between treatments (one-way ANOVA, p<0.05; n=3).

3.3 Invertebrates

Meiofauna

No statistical differences were found between meiofauna associated with both leaves (one-way ANOVA: $F_{(1,10)}$=0.31; p=0.59) (Fig.9). Eight taxonomical groups were found associated with both leaves: Nematode, Copepod, Polychaeta, Bivalvia and Turbellaria; Cladocera and Gastropoda were only found in alder while

Ostracoda was only present in poplar (Fig.10). After analyzing the contribution of each taxonomical group, nematodes seem to be present in higher proportion.

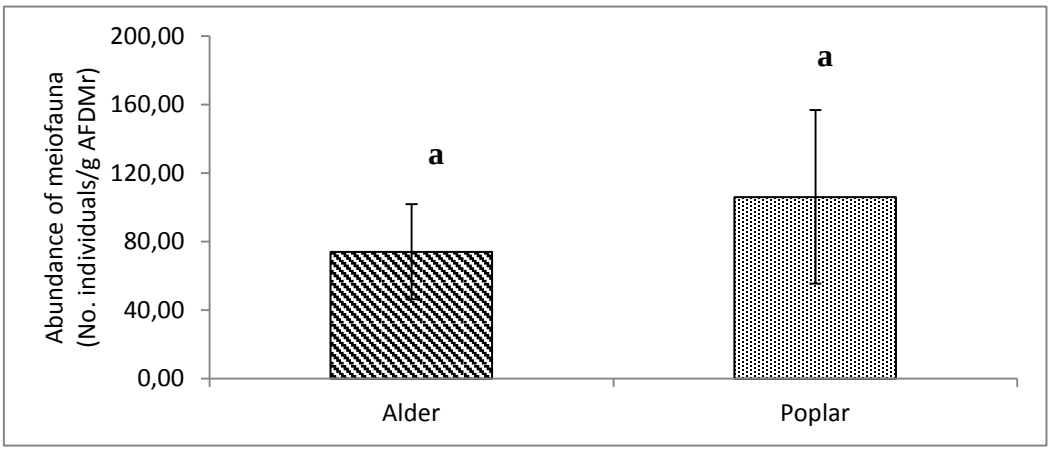

Fig. 9: Abundance of meiofauna (no. individuals/g AFDMr) found in alder and poplar after 21 days of exposure at our experimental site. The values correspond to the mean ± SD. Different letters indicate differences between treatments (one-way ANOVA, $p<0.05$; n=3).

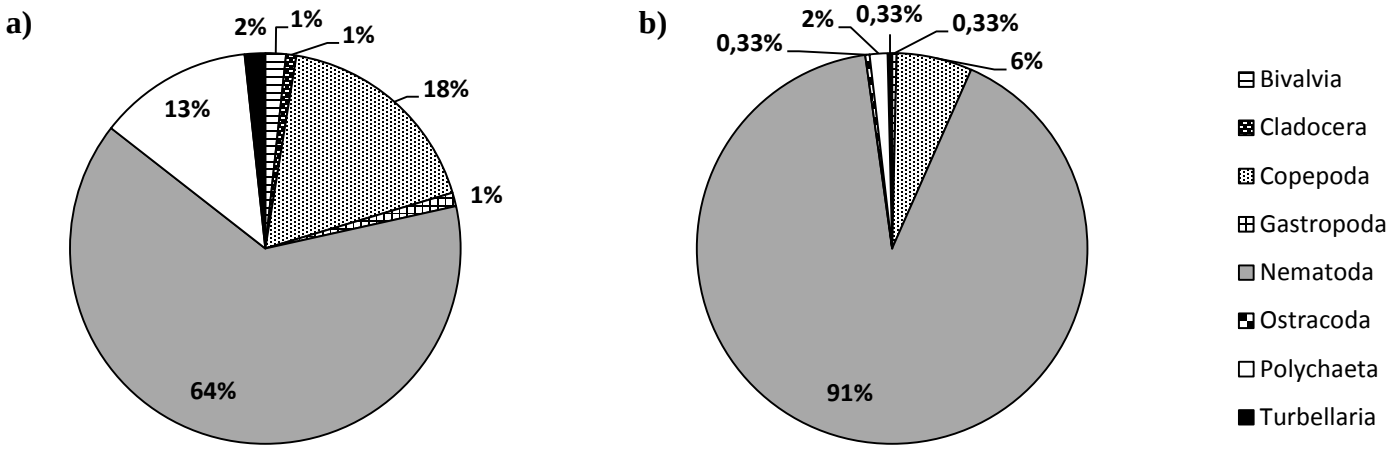

Fig. 10: Pie charts of the percentage of meiofauna abundance found for (a) alder and (b) poplar; n=3.

Macroinvertebrates

Invertebrates found in CM bags of both leaf species were almost inexistent. We found 3 individuals in alder leaves and 12 individuals in poplar leaves (Fig. 11).

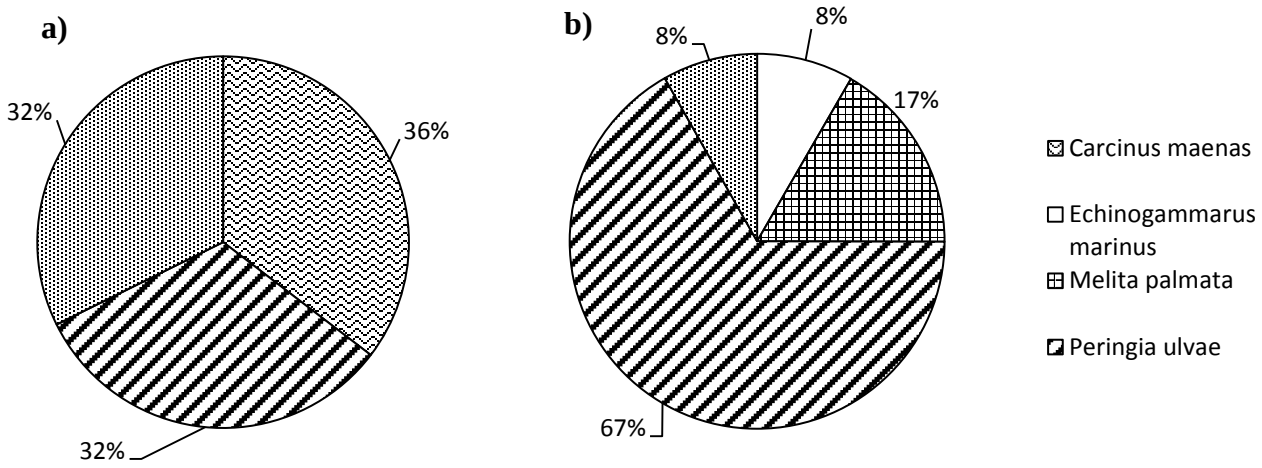

Fig. 11: Pie charts of the percentage of invertebrate abundance found for (a) alder and (b) poplar; n=3.

- CHAPTER 4 -

Discussion

4 DISCUSSION

A large body of literature exists on decomposition of leaves in streams (Abelho, 2001) but very few studies were made in the fluvial-estuarine transitional area (Lecerf et al., 2008; Fuentes-Cid et al., 2014 & 2015). With this study, we tried to contribute to fill this gap.

The first stage of decomposition starts upon immersion. The characteristics of the leaves change and, in our case, all the parameters followed, more or less, the pattern expected. The percentage of organic carbon (C) decreases with decomposition, as expected. A lot of the C that goes into the water is dissolved, and supports the respiration of suspended microorganisms and some microbes directly associated with the leaves (Howarth & Fisher, 1976). With leaching, the soluble compounds of the leaf (e.g. phenols, phosphorous and nitrogen) are released into the water (Abelho, 2001). Some phenols are highly soluble components of the leaves that remain present after death of the leaf; they also are a line of defense against herbivores and pathogens (Covelo & Gallardo, 2001; Bärlocher & Graça, 2005). They rapidly leachate when submerged in water but their presence may delay the microbial colonization and degradation of leaf litter (Bärlocher & Graça, 2005). In our experiment, alder leaves had their N contents significantly increased after 21 days, which was not the case in poplar leaves. This might have happened due to the higher recalcitrance of poplar, that may favor a bacterial (rather than fungal) colonization. Fungi are known to incorporate N from the water column increasing leaves N contents, turning the leaves more palatable for invertebrates (Sridhar & Bärlocher, 2000).

Previous studies (Graça et al., 1993) state that litter decomposers usually prefer to consume leaves with high amounts of nitrogen and lower toughness, which in our case corresponds to the alder leaves. However, our results in terms of decomposition rates (*k*) show no significant differences between leaf species even though, poplar leaves seem to have a tendency to decompose faster than alder (*k* values are higher in poplar), in both mesh bags. This goes against our first hypothesis that stated that the less recalcitrant leaf (alder) would decompose faster. This might be explained if phosphorus is a limiting factor in this area. It might be considered more important for the decomposers, which leads to higher decomposition rates in the leaf with greater phosphorus contents (poplar). Other than that, the high amount of bacteria found in these leaves (eventually more adapted to these transitional waters than fungi) may attract the meiofaunal community stimulating decomposition (Coull, 1999).

Our results show that the Oxygen consumption was not different between leaf types but, it is statistically higher in CM bags for both alder and poplar. This probably occurs due to an easier oxygenation in these bags, which promotes higher microbial metabolic activity. This activity was translated in a non-significant increase of leaf decomposition rates in alder; however, such higher activity of decomposers (likely bacteria), eventually translated in higher or more efficient enzymatic degradative capacity, seem to contribute to accelerate leaf decomposition on poplar. These results in the CM bags impelled us to discover who are the real causers of these higher consumptions.

Working on low order streams always revealed that fungi have a major role in the decomposition of leaf material; not only helping to degrade the leaf itself, but also acting as an important food source for aquatic invertebrates to feed on, thereby increasing the rate of breakdown of the leaves (Gessner et al., 1999; Bärlocher, 2005; Canhoto & Graça, 2008). One way to detect the presence of fungi is to identify the presence of ergosterol on the leaf, which is the main sterol present in the cells or mycelial membranes (Ricardo, 2013). However, our results showed, unexpectedly, only vestigial presence of ergosterol in alder and poplar. Even though we found some spores in the leaves of the coarse mesh bags, they were low in numbers and fungal richness was also low. The only spores found on the leaves were from one terrestrial fungus, *Fusarium* sp. (present in both leaf species) and two aquatic hyphomycetes, *Heliscus lugdunensis* and *Tetrachaetum elegans* (present in alder and poplar, respectively).

The genus *Fusarium* is considered a common ground (soil-borne) fungus, usually associated with plant roots and debris (Mandeel, 2006; Llamas et al., 2008). Its occurrence in stream submerged leaves (Chamier et al., 1984), tidal marshes, stagnant water and fluvial waters tend to support its role as decomposer of leaves and branches of surrounding trees in these areas subjected to immersion and emersion periods (Wylloughby & Archer, 1973; Bärlocher & Kendrick, 1974; Chamier et al., 1984). In fact, in a study performed by Llamas et al. (2008) with several species of the *Fusarium* genus, they found that, although some species see its gemination decreased or disabled with increasing levels of salinity, some other species are positively affected with high values of NaCl. The authors confirm the possibility of some species of *Fusarium* being able to develop a saprophytic lifestyle in salty waters. This species physiological capabilities and long term

colonizer (Wylloughby & Archer, 1973) may justify its presence in 21 days conditioned leaves. Other than this, the spores of *Fusarium* sp. might have also simply attached itself to the leaves after abscission, when they fell on the ground (Bärlocher, 1992), not having any connection to the estuary. Moreover, it's also possible that our bags have been exposed outside of water in some moment, allowing the attachment of the spores, or even through an event of rain and soil runoff that enables the seepage of terrestrial spores onto the bags.

Tests on the effects of salinity on mycelial growth indicate that species of aquatic hyphomycetes are able to grow in salt-rich environment (Byrne & Jones, 1975; Simões et al., submitted); their sensitivity is species specific.

Both *H.lugdunensis* and *Tetrachaetum elegans* (found in alder and poplar, respectively), are commonly found in streams belonging to the catchment area of the Mondego river. Their presence in the area may result from spore dispersal in the water column or from air dispersal: these species can release spores in the air, allowing long range distribution (Webster, 1992; Laitung et al., 2004). This ability of discharging spores in the air, allied with the fact that our study site has areas of intertidal flats that are exposed at low tides (Marques et al., 2013) (consequently exposing the leaf bags) might facilitate the colonization of these species in our leaves. These species somatic growth has been shown to tolerate salinity, particularly *H. lugdunensis* which is not affected by the presence of NaCl in the growth medium; in the case of *T. elegans* EC50 for growth is 14g/L NaCl (Simões et al., submitted) which is in the range of the values found in our study site. Although sporulation of both species proved to be more sensitive to salinity than their growth, mycelium sporulation might have been allowed due to the oscillation in salt levels in this transitional area.

We found no differences in sporulation rates between leaf species, but there seems to exist a tendency to have higher rates in poplar leaves. This might occur due to poplar greater toughness, which facilitates fungal mycelium growth, consequently leading to higher sporulation. After 21 days conditioning, alder is possibly over-conditioned, which may limit mycelial growth and spore production.

Bacteria seem to play a big role in decomposition of organic matter in estuarine areas (Heip et al., 1995; Crump et al., 1998 & 1999). In this study, the abundance of this prokaryotic group was higher in the more recalcitrant leaf. This may be related with the fact that, after 21 days, poplar offers a more stable and still usable substratum (e.g. leaf veins, some recalcitrant tough mesophyll) for the bacterial communities; it is generally recognized that bacteria play a key role in the decomposition of more recalcitrant material being of high importance in later stages of leaf degradation (Wright & Covich, 2005).

In the evaluation of meiofauna, we found individuals belonging to the taxonomic groups of Nematode, Copepod, Polychaeta, Bivalvia, Turbellaria, Cladocera, Gastropoda and Ostracoda. Even though all of them exist in fresh and salty water, Nematodes are usually the most abundant in aquatic ecosystems (Coull, 1999; Hourston et al., 2011; Alves et al., 2013); the same pattern was observed in the meiofauna found in both leaf species. Although our results showed no statistical differences in meiofauna abundance between leaves, there is also a higher tendency for them to appear in poplar leaves. This could happen not only due to the higher abundance of bacteria in these leaves, but also due to the greater support and stability that poplar may offer due to its toughness in comparison with alder leaves. The seemingly tendency for nematodes to appear in higher proportion in our study

site might be explained by that fact that most nematodes feed primarily on bacteria which are abundant in our study zone; furthermore, the second group with higher percentage – copepods – feed mainly on microphytobenthos whose occurrence might be limited by the turbulence and depth of the estuarine zone in our study area, which possibly results in fewer individuals of this taxonomical group (Coull, 1999; Hourston et al., 2011). These explanations should, nevertheless, be faced with prudence and need further investigation.

The macroinvertebrates found (*C. maenas*, *E. marinus*, *M. palmata*, *P.ulvae* and *S. rugicauda*) in our samples were extremely low in numbers, which suggests that leaves may constitute an unimportant food source for this group, being leaf decomposition a dominant microbial-mediated process in this area. However, our bags were incubated in the margins of the river and were subjected to cycles of immersion and dehydration; such oscillations may have limited the invertebrates' access to the leaves. On the other hand, the bags may also constitute a stable substratum providing shelter and camouflage from predators. Other than that, the presence of these invertebrates in the leaf bags may be accidental. Nevertheless, the feeding behavior of some species may suggest that leaves by themselves may constitute an important food source for these invertebrates. For instance, *Peringia ulvae* is a periphyton grazer that feeds selectively on diatoms and fungal hyphae (Sousa, 2013) and laboratorial tests indicate that the isopod *Sphaeroma rugicauda* may feed on leaves and/or its biofilm (Marsden, 1979; Willis & Heath, 1985). Conversely, some of these invertebrates might be slowing the process of degradation of the leaves; for example, *C. maenas* is known to prey on meiofauna

(Grosholz et al., 1996) and *E. marinus* might feed on isopods (Dick et al., 2005) like *S. rugicauda* (Castañeda & Drake, 2008).

According to the RCC, collectors dominate in these transitional waters. Our study tends to support the point of view that shredders and grazers may have a limited degradative role of leaves entering the stream and remaining in the margins. Although the tolerance of fungi to salt is recognized, in our study, leaf litter biotic decomposition seems to be mainly promoted by bacteria and by a still largely unknown meiofauna. The use of multiple isotopic tracers may help to clarify this issue in these environments (McCallister et al., 2004). We cannot rule out the importance of abiotic factors such as salinity, tides and turbulence (Mao et al., 2004; Fuentes-Cid et al., 2015) as drivers of leaves degradation, even with the mesh bags reducing some of the mass losses from abrasion (Young et al., 2008).

As it was already stated, few studies have been made that characterize and try to understand leaf litter decomposition in transitional waters. Our work only scraped the surface of what is yet to discover about the real importance (quantitative and qualitative) of leaves in the fluvial-estuarine area and the recycling of nutrients. For following works, we suggest a study during the course of the seasons of the year to account for different temperatures, and overall climate and also throughout a gradient of salinity so as to characterize the microbial communities. Laboratory tests portraying the fluvial-estuarine conditions, with invertebrates collected in the area to assess their feeding response to leaves seem also necessary.

There are still many possible studies to be made in order to access and help comprehend the recycling of nutrients in these highly productive and agitated

systems and how they influence the aquatic trophic chains in these areas, allowing a good management of the ecosystem.

- CHAPTER 5 -

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