Universitat de Girona

# LEAF LITTER DECOMPOSITION IN MEDITERRANEAN STREAMS: MICROBIAL PROCESSES AND RESPONSES TO DROUGHT UNDER CURRENT GLOBAL CHANGE SCENARIO

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Juanita Mora Gómez

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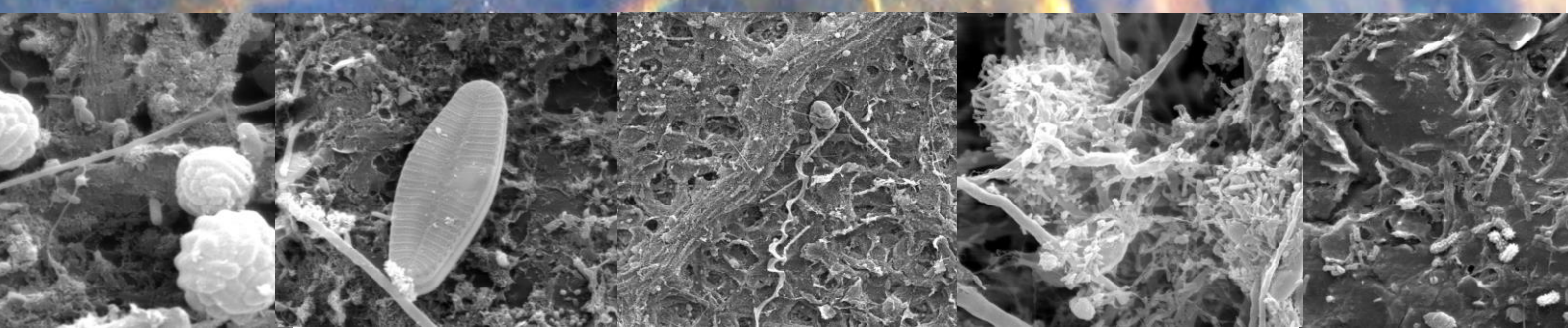

Universitat de Girona
Institut d'Ecologia Aquàtica

DOCTORAL THESIS

# LEAF LITTER DECOMPOSITION IN MEDITERRANEAN STREAMS: MICROBIAL PROCESSES AND RESPONSES TO DROUGHT UNDER CURRENT GLOBAL CHANGE SCENARIO

Juanita Mora Gómez
2014

DOCTORAL PROGRAMME IN EXPERIMENTAL SCIENCES AND SUSTAINABILITY

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This manuscript has been presented to opt for the doctoral degree from the University of Girona

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WE DECLARE:

That the thesis entitled "Leaf litter decomposition in Mediterranean streams: microbial processes and responses to drought under current global change scenario", presented by Juanita Mora Gómez to obtain a doctoral degree, has been completed under my supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, I hereby sign this document.

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Girona, Nov 2014

*A la ecología, con la esperanza que sea un granito de arena para el entendimiento del mundo que habitamos*.

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In this thesis I present four scientific papers from which I am the first author, and one in which I am the second author. Four articles are in preparation and one under revision. The references are cited below:

- **Mora-Gómez, J**., Elosegi, A., Mass-Marti, E. & A. M. Romaní. Factors controlling seasonality in leaf litter breakdown for a Mediterranean stream. Under revision Freshwater Science.

- **Mora-Gómez, J**., Elosegi, A., Duarte, S., Cássio, F., Pascoal, C. & A. M. Romaní. Dynamic microbial assemblages and enzyme activities throughout leaf litter decomposition in a Mediterranean stream. In prep.

- **Mora-Gómez, J**., Elosegi, A., Boix, D., Duarte, S., Cássio, F., Pascoal, C. & A. M. Romaní. Summer drought affects autumn leaf decomposition in streams. In prep.

- **Mora-Gómez, J.,** Duarte, S., Cássio, F., Pascoal, C. & A. M. Romaní. Emersion affects leaf litter microbial processing in a pristine temperate stream. In prep.

- Duarte, S., **Mora-Gómez, J.,** Cássio, F., Pascoal, C. & A. M. Romaní. Eutrophication alters responses of stream-dwelling microbial decomposers to drought. In prep.

**CONTENTS**

**SUMMARY**

Mediterranean intermittent streams are systems exposed to large flow and temperature fluctuation with a distinctive drought period where the hydrological connectivity is seasonally disrupted. Combined effects of land-use and climate change may determine more extreme environmental variations, increasing drought duration and intensity in naturally intermittent streams, as well as transforming permanent streams in temporaries. Litter breakdown is a pivotal ecosystem function in headwater streams, where it fuels food webs and controls the carbon flux. Microorganisms, fungi and bacteria, play a fundamental role in leaf litter processing hence they colonize the leaves and degrade leaf compounds through their enzyme activities, enriching and conditioning litter to invertebrates. Breakdown rates and microbial processing are highly dependent on environmental characteristics, and can therefore suffer strong seasonal variation under the particular harsh conditions of intermittent Mediterranean streams. Furthermore, summer drought period characteristic of this kind of ecosystems might potentially affect leaf litter processing through several ways, such as direct exposition of leaves to strong solar radiation and high temperature during dry period, pool conditions and emersion-immersion cycles of decomposing leaves.

The main objective of this thesis was to elucidate the principal environmental controlling factors and microbial mechanisms involved in litter decomposition in Mediterranean intermittent streams. More specifically, it was aimed to assess the seasonal fluctuation, microbial dynamics and drought impact on litter processing in order to understand the possible consequences of global change in this kind of ecosystems. Additionally, we tested the drought disturbance in Atlantic permanent streams non-naturally exposed to drying conditions by assessing the emersion effect on decomposing leaves; and evaluated the possible interaction effect of emersion and nutrient enrichment in these systems. To achieve our aims we performed several litterbag experiments with *Populus nigra* L. leaves, in three low-order streams: an intermittent Mediterranean stream located in the North-east of Spain (Fuirosos) and two natural permanent streams located in the North of Portugal: the Oliveira (oligotrophic stream) and the Este (eutrophic stream).

In the Fuirosos stream, we conducted an annual monitoring (only wet period) of total litter breakdown (coarse mesh) and microbial mediated breakdown (fine mesh); concurrently we studied the structural and functional dynamics of microorganims over poplar leaves decomposition in time. In addition, summer drought effect on autumn leaves decomposition was tested through an experiment where leaves were exposed to simulated summer drought conditions for 79 days, and were then immersed in the Fuirosos stream. Litter breakdown process of drought-exposed leaves was followed for 37 days and compared with the processing of non-exposed leaves. Finally we conducted a similar simulated-drought experiment but in two permanent Atlantic streams differing in the trophic status - Oliveira and Este. The experiment consisted in exposing poplar leaves to different duration of emersion conditions (7, 14 and 21

days), and evaluating decomposition and microbial attributes (microbial biomass, extracellular enzyme activities, and microbial diversity and assemblage structure) in both streams.

Based on the results from the Fuirosos stream, we demonstrated that in low order Mediterranean streams, total litter breakdown varied through wet period and this was mainly explained by seasonal changes in temperature, conductivity, and flow velocity. Despite microbial decomposers activity seemed to lead total litter breakdown, a differential microbial and invertebrate sensitivity to environmental parameters modulated seasonal responses. Microbial breakdown was particularly sensitive to temperature and water quality, while invertebrate breakdown was primarily associated with current velocity. Therefore, leaves were processed faster in spring and early summer, mainly favoured by flow stability and higher temperatures, but a lower water quality in summer due to severe flow reduction inhibited microbial and invertebrate breakdown in spite of having the highest temperatures.

The insight on the microbial leaf litter dynamics for all decaying processes analysed throughout the wet period showed that poplar microbial processing might be separated into two stages: initial and middle-late stage. In the initial stage (until 75% leaf mass remaining), microbes are starting leaf colonisation and enzyme activities and biomass presented low values. In this stage is when highest proportion of bacterial biomass in relation to fungal biomass was observed. Characteristic initial fungal and bacterial assemblages were formed in this first stage and related to a greater use of simple polysaccharides and lignin than cellulose compounds. In the middle-late stage, fungal and bacterial biomass increased but the percentage of bacterial biomass was reduced to less than 3%, showing the expected header role of fungi during the breakdown process. In this stage, both bacterial and fungal assemblages were related to the increasing of all measured extracellular enzyme activities linked to cellulose, hemicellulose and lignin decomposition. β-xylosidase and phenol oxidase showed the highest enzyme efficiency to decompose leaves suggesting that decomposition of hemicellulose and lignin might be key steps for degradation of poplar leaves.However, this described process also showed seasonal effects as well as being modulated by the presence of invertebrates. The relationships between leaf mass loss, enzyme activities and microbial biomass indicated that in spring a highly efficient fungal-dominated microbial community controlled the process, whereas in summer, bacteria were favoured and litter decomposition was strongly limited by lignin degradation. Invertebrate presence affected both bacterial and fungal assemblages, and bacteria growing seemed to be further stimulated by invertebrates.

The specific study of the summer drought period indicated that exposition of fallen leaves to characteristic summer drought conditions of intermittent Mediterranean stream has critical consequences in litter processing once the water returns in autumn, affecting leaf quality, breakdown mechanisms and decomposer and detritivore assemblages, although breakdown velocity might remain unaffected. Summer drought increased nitrogen, total fibre, lignin and cellulose content of poplar leaves, which was reflected in shifts of fungal and bacterial assemblages, and in turn, was related with higher cellulose-degrading enzyme activity and lower lignin-degrading enzyme activity. Furthermore, drought exposition of leaves reduced

variability among replicates of all the studied community parameters (biomass and assemblages) for bacteria, fungi and macroinvertebrates, suggesting that communities involved in decomposition were functionally and structurally homogenized when leaves were pre-exposed to drought.

On the other hand, the experiment performed in an Atlantic “pristine” permanent stream showed that under different length of exposure to emersion (0 –control–, 7, 14 and 21 days), litter breakdown, microbial activities and microbial assemblages on poplar leaves were impacted. Decomposition rate and cellulose and organic phosphorus compounds decomposition were progressively delayed with increasing desiccation time, which may be related to slower terrestrial processing velocities. Microbial assemblages were modified due to drying exposition but higher sensitivity was found for sporulating fungal assemblages, and for bacterial assemblages than for total fungal assemblages. Hemicellulose and lignin degradation were more sensitive to emersion than cellulose and organic phosphorus compounds degradation, while bacterial biomass showed greater resistance to drying conditions. We observed fungal assemblages in leaves exposed to emersion to be related with proportionally lower lignin and hemicellulose degradation capacities.

Comparing the oligotrophic to the eutrophic permanent stream we observed a differential response to emersion disturbance. In general, lower leaf decomposition rates were observed in the eutrophic than in the oligotrophic stream, as well as significant basal differences in microbial activity and structure. Emersion strongly affected all functional measures analysed, but these effects appear to be different in the two streams. While leaf decomposition and enzyme activities were more sensitive to emersion exposure at the most oligotrophic site, fungal sporulation and microbial biomass were more affected at the most eutrophic site. Overall, the oligotrophic stream showed to be more sensitive to emersion disturbance than the eutrophic stream, which might had been a direct effect of leaves carrying out different initial microbial assemblages, which in turn presented different functional stabilities to the stress imposed by emersion of leaves.

Overall, the thesis results show that litter decomposition in intermittent Mediterranean streams is affected by annual environmental variation mainly related to changes in microbial enzyme eficiencies, and possible seasonal fluctuation of shredders, and highlights the relevance of drought period in these ecosystems. Microbial assemblages and functioning appear to be sensitive to drought, although this may not always result in changes on breakdown rates. In permanent streams drought also affects microbial activity and structure but microbial communities from nutrient enriched streams seem to be more resistant to drought disturbance.

**RESUMEN**

Los ríos mediterráneos intermitentes son sistemas expuestos a una amplia fluctuación en temperatura y caudal a través del año. En el verano, estos ríos pueden afectarse por las altas temperaturas y la baja precipitación, y presentar un periodo de desconexión hidrológica generando que el rio se seque. El efecto combinado del uso de la tierra y el cambio climático que se conjugan en la actualidad, puede determinar que las variaciones en ríos mediterráneos sean más extremas y que aumente la duración e intensidad del periodo de sequía en el río. Del mismo modo este escenario actual puede generar que ríos que naturalmente son permanentes, sean expuestos a este tipo de disturbio. La descomposición de la hojarasca es un proceso fundamental en ríos de cabecera ya que es parte del reciclaje del carbono y ayuda a mantener las cadenas tróficas. Los microorganismos, principalmente hongos y bacterias, juegan un papel determinante en el proceso de descomposición. Ellos ayudan a degradar la hojarasca a través de sus actividades enzimáticas extracelulares, enriqueciéndola y condicionándola para que sea más nutritiva para los invertebrados acuáticos que la consumen.

La velocidad a la que la hojarasca es degradada y la actividad de los microorganismos sobre la hoja dependen en gran medida de las características ambientales en el río, por lo que pueden ser afectados por los fuertes cambios estacionales que se observan en ríos mediterráneos. Además, el periodo de sequía en verano, típico de estos sistemas, pueden afectar potencialmente el proceso de descomposición a través de diferentes condiciones, tales como: los cambios en la calidad del agua y la reducción del hábitat durante la formación de balsas al inicio del proceso de secado del río, la exposición directa de la hojarasca a una fuerte radiación solar y altas temperaturas durante el tiempo que el río permanece seco, y la emersión y/o re-inmersión de la hojarasca en descomposición, que se genera principalmente durante la reducción del caudal.

El objetivo principal de esta tesis es esclarecer los factores ambientales determinantes y los mecanismos microbianos involucrados en la descomposición de la hojarasca en ríos intermitentes mediterráneos. De manera específica, esta tesis evaluó la fluctuación estacional de la descomposición, las dinámicas microbianas y el impacto de la sequía sobre el procesamiento de la hojarasca, con el fin de entender las posibles consecuencias del cambio global en ríos intermitentes mediterráneos. Adicionalmente, también se evaluó el potencial efecto de la sequía en ríos permanentes, los cuales no están naturalmente expuestos a la extrema reducción del caudal, analizando el efecto de emersión de la hojarasca sobre su descomposición en un sistema inalterado, y evaluando el efecto conjunto con el enriquecimiento de nutrientes en un río eutrofizado. Para cumplir con los objetivos planteados se realizaron varios experimentos con hojas recién caídas de *Populus nigra* L. (álamo negro), usando la técnica de bolsas de malla, en tres ríos de bajo orden: un río intermitente mediterráneo localizado al noreste de España (Fuirosos), y en dos ríos permanentes atlánticos localizados al norte de Portugal, uno oligotrófico (Oliveira) y uno eutrófico (Este).

En Fuirosos se llevó a cabo un monitoreo anual durante el periodo húmedo (periodo en el que el río lleva agua), midiendo la descomposición total (malla gruesa) y la descomposición mediada por microorganismos (malla fina) de las hojas del álamo negro. De manera conjunta, a lo largo de los procesos de descomposición evaluados en el monitoreo, se estudiaron las dinámicas de aspectos funcionales y estructurales de las comunidades microbianas. Además, se estudió el efecto de la exposición de la hojarasca a las condiciones extremas del verano (alta temperatura y radiación solar) sobre la descomposición en otoño, cuando el agua retorna al río. Este efecto se evaluó por medio de un experimento combinado, donde la hojarasca fue primero expuesta a condiciones simuladas de sequía a lo largo de 79 días, y luego se siguió el proceso de descomposición en el río por 37 días, comparando el procesamiento de las hojas expuestas con hojas no expuestas. Finalmente, a través de un experimento similar al realizado en Fuirosos, se estudió el efecto de la sequía en los ríos permanentes Atlánticos, comparando un río eutrofizado con uno en condiciones de poca alteración. El experimento consistió en colonizar hojas de álamo negro en cada río por una semana, luego exponerlas a condiciones secas durante diferentes tiempos de emersión (7, 14 y 21 días), y luego volverlas a su respectivo río. A lo largo de todo el experimento se midió la pérdida de peso y aspectos de la comunidad microbiana.

Los resultados de Fuirosos mostraron que en ríos intermitentes mediterráneos, la descomposición de la hojarasca varía estacionalmente a lo largo del periodo húmedo y que los factores que más explican esta variación son: la temperatura, la conductividad y la velocidad del agua. A pesar de que la contribución de los microorganismos en el proceso total de descomposición fue constantemente mayor, se observó que la estacionalidad encontrada para la descomposición pudo estar mediada por la respuesta diferencial de microorganismos e invertebrados a los cambios estacionales. La descomposición mediada por microorganismos se vio más influenciada por cambios en temperatura y calidad del agua (pH, oxígeno y conductividad), mientras la descomposición mediada por invertebrados estuvo más relacionada con cambios en la velocidad del agua. De esta manera, las hojas de álamo negro fueron procesadas de manera más eficiente en primavera e inicio del verano, principalmente favorecido por la estabilidad del caudal y las altas temperaturas que se presentaron en esta estación, sin embargo, en verano la baja calidad del agua asociada con la reducción del caudal, pareció inhibir la actividad de microorganismos e invertebrados, a pesar del incremento esperado por las altas temperaturas.

El análisis del procesamiento microbiano de la hoja de álamo negro a lo largo de la fase húmeda del río, mostró que su descomposición se puede separar en dos fases, una inicial y una media-tardía. La fase inicial cubrió las primeras semanas (hasta el 75% de peso remanente), presentando valores bajos de biomasa microbiana y actividades enzimáticas. En esta fase se observó la mayor contribución de bacterias en relación a la biomasa de hongos. Además, se formaron asociaciones de hongos y bacterias distintivas, las cuales estuvieron relacionadas con una mayor producción de enzimas para degradar polisacáridos y lignina, que para degradar celulosa. En la fase media-tardía, la biomasa de hongos y bacterias aumentó

pero la contribución de las bacterias a la biomasa microbiana total se redujo a menos del 3%, lo que evidenció un rol predominante de los hongos en esta fase, lo que se esperaría para el proceso de descomposición. En esta segunda fase, también se observaron distintivas asociaciones de hongos y bacterias, las cuales estuvieron relacionadas con un incremento de las enzimas responsables de la degradación de celulosa, hemicelulosa y lignina. Teniendo en cuenta todo el proceso, la eficiencia en descomponer la hoja fue mayor para la β-xilosidasa y fenol oxidasa, sugiriendo que la degradación de hemicelulosa y lignina pueden ser pasos determinantes en la descomposición de las hojas del álamo negro. Los patrones en el proceso de descomposición observado fueron afectados por los cambios estacionales y la presencia de invertebrados. La relación entre la pérdida de peso, las actividades enzimáticas y las biomasas microbianas indicaron que en primavera el proceso de descomposición es dominado por una comunidad altamente eficiente, principalmente dominada por hongos, mientras en verano, las bacterias se ven favorecidas, aumentando su contribución y el proceso se ve limitado por la degradación de lignina. Bajo la presencia de los invertebrados las asociaciones de hongos y bacterias se vieron afectadas y el crecimiento bacteriano pareció estimularse.

El estudio realizado para evaluar el efecto de la exposición de las hojas a las condiciones de sequía en verano (alta radiación solar y temperaturas) en ríos intermitentes mediterráneos, mostró que la exposición de las hojas tiene consecuencias claras en el procesamiento de la hojarasca cuando son sumergidas, una vez el río ha recuperado su caudal en otoño. Los efectos se observaron en la composición de las hojas, los mecanismos microbianos enzimáticos y las asociaciones de microorganismos y detritívoros, sin embargo la velocidad de descomposición no se vio afectada. La exposición de las hojas de álamo negro al verano aumentó su contenido de nitrógeno, fibra, lignina y celulosa, lo cual se vio reflejado en cambios de las asociaciones de hongos y bacterias. Del mismo modo, estos cambios se relacionaron con una mayor actividad enzimática para degradar celulosa y una menor actividad para degradar lignina. Además, la exposición a las condiciones extremas del verano redujeron la variabilidad entre réplicas de todas los parámetros de la comunidad estudiados (biomasas y asociaciones de especies), tanto en bacterias, como en hongos y en macroinvertebrados, sugiriendo que las comunidades encargadas de la descomposición son funcional y estructuralmente homogenizadas cuando las hojas son pre-expuestas a la sequía.

Por otra parte, el experimento realizado en un río permanente atlántico con baja alteración humana, mostró que diferentes tiempos de emersión (0-control-, 7,14 y 21 días) de las hojas del álamo negro en descomposición, afectan la tasa de degradación y las características estructurales y funcionales de las comunidades microbianas. La velocidad de descomposición y la degradación de celulosa y compuestos orgánicos de fósforo fueron progresivamente desaceleradas con el aumento en el tiempo de emersión, lo cual se puede relacionar con un procesamiento microbiano de la hoja más lento en condiciones terrestres. Las asociaciones de microorganismos fueron modificadas por la emersión, aunque se observó una mayor sensibilidad de las asociaciones de hongos que producen esporas y las de bacterias, que en el total de taxones de hongos presentes en las hojas. La degradación de

hemicelulosa y lignina fue más sensible a la emersión que la degradación de celulosa y los compuestos orgánicos de fósforo, mientras la biomasa bacteriana mostró una mayor resistencia a la emersión. Los cambios en las asociaciones de hongos debidos a la emersión de las hojas se relacionó con una degradación proporcionalmente menor de lignina y celulosa.

En la comparación entre el río permanente oligotrófico y el eutrófico, la respuesta a la emersión de las hojas en descomposición del álamo negro varió entre los dos ríos. En general en el río eutrofizado se observaron menores tasas de descomposición, así como diferencias significativas basales en la actividad y estructura de los microorganismos. La emersión de las hojas afectó de manera importante las medidas funcionales analizadas, pero estos efectos parecieron ser diferentes entre los dos ríos. Mientras la descomposición y las actividades enzimáticas fueron más sensibles a la emersión en el río oligotrófico, la esporulación de los hongos y las biomasas microbianas fueron más afectadas en el río eutrófico. En general, el río oligotrófico mostró ser más sensible al efecto de la emersión de las hojas que el río eutrofizado, lo que pudo ser debido a un efecto directo de las diferentes asociaciones microbianas iniciales formadas en las hojas, las que a su vez presentaron diferentes estabilidades funcionales al estrés determinado por la emersión.

De manera global, los resultados de esta tesis muestran que la descomposición es afectada por la variación ambiental anual de los ríos intermitentes mediterráneos, a través de cambios en las eficiencias enzimáticas de los microorganismos descomponedores, así como posiblemente en relación con fluctuaciones estacionales de los invertebrados trituradores. Además, se remarca la relevancia del periodo de sequía en estos ecosistemas, el cual determina cambios importantes en las asociaciones de microorganismos descomponedores y su funcionamiento, aunque no siempre esto se vea reflejado en cambios en la velocidad de descomposición de la hoja. Del mismo modo, en ríos permanentes las posibles consecuencias de la sequía medidas a través del efecto de la emersión de las hojas, también mostraron una gran sensibilidad de las comunidades microbianas en su funcionamiento y estructura, sin embargo las comunidades de ríos con aumento de nutrientes fueron más resistentes a este disturbio.

## RESUM

Els rius mediterranis intermitents són sistemes exposats a una àmplia fluctuació de temperatura i cabal al llarg de l'any. A l'estiu, aquests rius poden veure's afectats per les elevades temperatures i baixa precipitació, i presentar un període de desconnexió hidrològica generant que el riu s'assequi. L'efecte combinat dels usos del sòl i el canvi climàtic que es dóna actualment poden determinar que les variacions en rius mediterranis siguin més extremes i que augmenti la durada i intensitat del període de sequera als rius. Així mateix aquest escenari actual pot generar que rius que naturalment són permanents, siguin exposats a aquest tipus de pertorbació. La descomposició de la fullaraca és un procés fonamental en rius de capçalera ja que forma part del reciclatge de carboni i ajuda a mantenir les cadenes tròfiques. Els microorganismes, principalment bacteris i fongs, juguen un paper determinant en el procés de descomposició. Aquests ajuden a degradar la fullaraca a través de les seves activitats enzimàtiques extracel·lulars, enriquint i condicionant la fulla per a que sigui més nutritiva per als invertebrats aquàtics que la consumeixen.

La velocitat de descomposició de la fullaraca i l'activitat dels microorganismes sobre la fulla depenen en gran mesura de les característiques ambientals del riu, i per tant es pot veure afectada pels intensos canvis estacionals que tenen lloc en rius mediterranis. A més a més, el període de sequera de l'estiu, típic d'aquests sistemes, pot afectar potencialment el procés de descomposició a través de diferents condicions que es generen principalment durant la reducció del cabal, tals com: els canvis en la qualitat de l'aigua i la reducció de l'hàbitat durant la formació de basses a l'inici del procés d'assecament del riu, l'exposició directa de la fullaraca a la forta radiació solar i elevades temperatures durant el temps que el riu és sec, i l'emersió i/o re-immersió de la fullaraca en descomposició.

L'objectiu principal d'aquesta tesi és d'esclarir els factors ambientals determinants i els mecanismes microbians involucrats en la descomposició de la fullaraca en rius intermitents mediterranis. De manera específica, aquesta tesi avalua la fluctuació estacional de la descomposició, la dinàmica dels microorganismes i l'impacte de la sequera sobre el processat de la fullaraca, amb la finalitat d'entendre les possibles conseqüències del canvi global en rius intermitents mediterranis. A més a més, aquesta tesi també avalua l'efecte potencial de la sequera en rius permanents, els quals no estan de forma natural exposats a l'extrema reducció del cabal, analitzant l'efecte de l'emersió de la fullaraca sobre la seva descomposició en un sistema inalterat, i avaluant l'efecte conjunt amb l'enriquiment de nutrients en un riu eutrofitzat. Per complir amb els objectius plantejats s'han realitzat diferents experiments amb fulles recent caigudes de *Populus nigra* L. (pollancre), utilitzant la tècnica de bosses de malla, en tres rius d'ordre baix: un riu intermitent mediterrani localitzat al nord-est de la Península Ibèrica (Fuirosos), i en dos rius permanents atlàntics localitzats al nord de Portugal, un d'oligotròfic (Oliveira) i un d'eutròfic (Este).

A Fuirosos es va realitzar un seguiment anual durant el període humit (període durant el qual el canal fluvial porta aigua) de la descomposició total (utilitzant bosses de malla grossa) i de la descomposició per part de microorganismes (utilitzant bosses de malla fina), de les fulles de pollancre. De manera conjunta es van estudiar les dinàmiques funcionals i estructurals de les comunitats microbianes durant els processos de descomposició avaluats durant el seguiment. A més a més, es va estudiar l'efecte de l'exposició de la fullaraca a les condicions extremes d'estiu (altes temperatures i radiació solar) en la descomposició durant la tardor, quan l'aigua torna a fluir al riu. Aquest efecte es va avaluar mitjançant un experiment combinat on la fullaraca es va exposar primer a condicions simulades de sequera durant 79 dies i després es va seguir el procés de descomposició al riu durant 37 dies, comparant el processament de les fulles exposades a la sequera amb fulles no exposades. Finalment es va realitzar un experiment similar al realitzat a Fuirosos, per tal d'avaluar l'efecte de la sequera en rius permanents atlàntics, un eutrofitzat i l'altre en condicions de poca alteració. L'experiment va consistir en colonitzar fulles de pollancre a cada riu durant una setmana, a continuació exposar-les a condicions seques durant diferents temps d'emersió (7, 14, i 21 dies), i després tornar les fulles als respectius rius. Durant tot l'experiment es va mesurar la pèrdua de pes i aspectes de la comunitat microbiana.

En base als resultats de Fuirosos es va trobar que en rius intermitents mediterranis, la descomposició de la fullaraca varia estacionalment durant el període humit i que els factors que més expliquen aquesta variació són la temperatura, la conductivitat i la velocitat de l'aigua. Malgrat que la contribució dels microorganismes en el procés total de descomposició fou sempre més elevada que la dels invertebrats, es va observar que l'estacionalitat de la descomposició podria ser resultat de la resposta diferencial de microorganismes i macroinvertebrats als canvis estacionals. La descomposició microbiana es va veure més influenciada per canvis de temperatura i qualitat de l'aigua (pH, oxigen i conductivitat), mentre que la descomposició per part de macroinvertebrats va ser més relacionada amb canvis en la velocitat de l'aigua. D'aquesta manera, les fulles de pollancre van ser processades de forma més eficient a la primavera i principis d'estiu, principalment afavorit per una estabilitat del cabal i les altes temperatures. Per altra banda, la baixa qualitat de l'aigua a l'estiu associada a la reducció del cabal, podria haver inhibit la descomposició microbiana i per part de macroinvertebrats, malgrat l'augment de l'activitat esperat per les altes temperatures.

L'anàlisi del processat microbià de les fulles de pollancre durant la fase humida, va mostrar que la seva descomposició es pot separar en dues fases, una inicial i una mitjana-tardana. La fase inicial incloïa les primeres setmanes fins al 75% del pes romanent de fullaraca, durant la qual la biomassa i les activitats enzimàtiques eren baixes. En aquesta fase es va observar una major contribució de bacteris en relació a la biomassa de fongs. A més a més, es van formar associacions de fongs i bacteris distints, els quals es van relacionar amb una major producció d'enzims involucrats en la degradació de polisacàrids i lignina per contra de menor producció d'enzims de degradació de cel·lulosa. Durant la fase mitjana-tardana la biomassa de fongs i bacteris va augmentar però la contribució dels bacteris a la biomassa microbiana total

es va reduir a menys del 3%, el que va evidenciar el paper predominant dels fongs que s'esperaria durant el procés de descomposició. Durant aquesta segona fase, es van observar també associacions distintives de fongs i bacteris, les quals es van relacionar amb un augment dels enzims responsables de la degradació de cel·lulosa, hemicel·lulosa i lignina. Tenint en compte tot el procés, l'eficiència en degradar la fulla va ser major per la β-xilosidasa i fenol oxidasa, suggerint que la degradació de l'hemicel·lulosa i lignina poder ser passos determinants en la degradació de les fulles de pollancre. Els patrons observats del procés de descomposició es van veure afectats pels canvis estacionals i per la presència de macroinvertebrats. La relació entre la pèrdua de pes, les activitats enzimàtiques i les biomasses microbianes indicaren que durant la primavera el procés de degradació de fullaraca és dominat per una comunitat altament eficient, principalment dominada per fongs. Per contra, a l'estiu els bacteris es veuen afavorits, augmentant la seva contribució i el procés es veu limitat per la degradació de lignina. Amb la presència de macroinvertebrats les associacions de fongs i bacteris es van veure afectades i el creixement bacterià es va veure estimulat.

L'estudi realitzat per a avaluar l'efecte de l'exposició de les fulles a les condicions de sequera de l'estiu (alta radiació solar i temperatura) en rius intermitents mediterranis, va mostrar que l'exposició de les fulles té conseqüències clares en el processat de la fullaraca un cop submergides al riu quan el riu ha recuperat el seu cabal a la tardor. Els efectes es van observar en la composició de les fulles, els mecanismes enzimàtics microbians i les associacions de microorganismes i detritívors, però la velocitat de descomposició no es va veure afectada per l'exposició a la sequera. L'exposició de les fulles de pollancre a les condicions d'estiu va fer augmentar els seu contingut de nitrogen, fibra, lignina i cel·lulosa, el qual es va veure reflectit en canvis en les associacions de fongs i bacteris. De la mateixa manera, aquests canvis es van relacionar amb una major activitat enzimàtica per a la degradació de cel·lulosa i una menor activitat per a la degradació de lignina. A més a més, l'exposició a les condicions de sequera estival van reduir la variabilitat entre rèpliques de tots els paràmetres de la comunitat avaluats (biomassa i associacions d'espècies), tant de bacteris com de fongs i macroinvertebrats, suggerint que les comunitats encarregades de la descomposició van ser homogeneïtzades tan funcional com estructuralment.

Per altra banda, l'experiment realitzat en un riu permanent atlàntic amb baixa alteració humana, va mostrar que diferents temps d'emersió (0-control-, 7,14 i 21 dies) de les fulles de pollancre en descomposició, afecten la taxa de descomposició i les característiques estructurals i funcionals de les comunitats microbianes. La velocitat de descomposició i la degradació de cel·lulosa i compostos orgànics de fòsfor van ser progressivament desaccelerats amb l'augment del temps d'emersió, el qual es pot relacionar amb un processament microbià de la fulla més lent en condicions terrestres. Les associacions microbianes van ser modificades per l'emersió, encara que es va observar una major sensibilitat de les associacions de fongs productors d'espores i les de bacteris que per al total de tàxons de fongs presents a les fulles. La degradació d'hemicel·lulosa i lignina va ser més sensible a l'emersió que la degradació de cel·lulosa i de compostos orgànics de fòsfor, mentre que la biomassa bacteriana va mostrar

una major resistència a la sequera de les fulles. Els canvis en les associacions de fongs deguts a l'emersió de les fulles es va relacionar amb una degradació proporcionalment menor de lignina i cel·lulosa.

En la comparació entre el riu permanent oligotròfic i l'eutròfic, la resposta a l'emersió de les fulles en descomposició de pollancre va ser diferent entre els dos rius. En general al riu eutròfic es van observar menors taxes de descomposició, així com diferències significatives basals en l'activitat i estructura dels microorganismes. L'emersió de les fulles va afectar de manera important les mesures funcionals analitzades, però aquests efectes van ser diferents entre els dos rius. La descomposició i les activitats enzimàtiques van ser més sensibles a l'emersió al riu oligotròfic, mentre que l'esporulació dels fongs i les biomasses microbianes van ser més afectades al riu eutròfic. En general, el riu oligotròfic va mostrar ser més sensible a l'efecte de l'emersió de les fulles que el riu eutròfic, fet que podria ser degut a un efecte directe de les diferents associacions microbianes inicials formades a les fulles, les que a la vegada van presentar diferents estabilitats funcionals a l'estrès determinat per l'emersió.

De manera global, els resultats d'aquesta tesi mostren que la descomposició es veu afectada per la variació ambiental anual dels rius intermitents mediterranis, a través de canvis en les eficiències enzimàtiques dels microorganismes descomponedors, així com possiblement en relació amb les fluctuacions estacionals dels invertebrats trituradors. A més a més, es remarca la rellevància del període de sequera en aquests ecosistemes, el qual determina canvis importants en les associacions de microorganismes descomponedors i el seu funcionament, malgrat no sempre això resulti en canvis de la velocitat de degradació de la fulla. Així mateix, en rius permanents les possibles conseqüències de la sequera mesurada a través de l'efecte de l'emersió de les fulles, també van mostrar una gran sensibilitat de les comunitats microbianes en el seu funcionament i estructura, malgrat que les comunitats en rius amb una elevada concentració de nutrients van ser més resistents a aquesta pertorbació.

# General Introduction

## RELEVANCE OF LEAF LITTER DECOMPOSITION IN STREAMS

Decomposition, or the catabolism of organic matter (OM) into its inorganic constituents, is essential for sustaining life on Earth, as it is the only process enabling massive recycling of chemical elements on a whole biosphere scale. Globally, between 80 to 90% of the matter from terrestrial and aquatic plant production is recycled through the detritus pathway rather than consumed by herbivores (Zimmer 2008, Gessner et al. 2010). Dead OM, or detritus, serves as substratum and food source for diverse microorganisms and detritus-feeding animals, thus influencing food web composition and dynamics, and increasing system stability and persistence (Moore et al. 2004).

In aquatic ecosystems, detritus has traditionally been divided into three broad size classes: 1) coarse particulate OM (CPOM, >1 mm), 2) fine particulate OM (FPOM, 0.5 µm-1 mm), and 3) dissolved OM (<0.5 µm, DOM), and it might come from both autochthonous and allochthonous sources (Allan and Castillo 2007). Autochthonous organic matter is originated within the aquatic ecosystems and includes dead macrophytes, animal faeces and dead biofilm material (Hanlon 1982), and also DOM released by living primary producers. On the other hand, allochthonous organic matter comes from terrestrial ecosystems and is mainly composed by leaves, stems, flowers, seeds and fruits, and logs (Benfield 1997, Pozo et al. 1997). The relevance of allochthonous organic matter is especially high in low-order forested streams, where the riparian cover strongly constrains autotrophic metabolism (Vannote et al. 1980, Wallace et al. 1999). Moreover, litter inputs into aquatic systems change depending on riparian vegetation and seasonality (Pozo et al. 1997, Abelho 2001, Tank et al. 2010).

Allochthonous CPOM gets into the stream channel through vertical or lateral inputs, and, once there, it is scoured downstream, transferred into living biomass, or transformed into FPOM or DOM through complex physico-chemical and biological processes, (Petersen and Cummins 1974, Webster and Benfield 1986, Gessner et al. 1999, Abelho 2001). In low order streams, CPOM is mainly constituted by leaves, that account for more than 60% of total litter fall (Abelho 2001, Table 1) and their fast processing makes them a fundamental carbon source (Gulis et al. 2008). However, wood is also a relevant component of CPOM due to its resistance to flood, allowing their longer time remaining in the system than leaves, and its decomposition significantly contributes to carbon cycle (Elosegi et al. 2007)

Table 1. Collection of data indicating total amounts of litter fall inputs per year and percentage of leaves inputs with respect to total litter fall into streams from different geographic areas. Selection from Abelho (2001).

| Location | Vegetation | Litter fall ($g\ m^{-2}\ year^{-1}$) | % of leaves | Reference |
|---|---|---|---|---|
| Denmark | Deciduous | 716 | 71 | (Iversen et al. 1982) |
| Central Finland | | 310 | 87 | (Haapala and Muotka 1998) |
| Central Portugal | Mixed deciduous | 715 | 63 | (Abelho and Graça 1998) |
| North Spain | Mixed deciduous | 759 | 66 | (Pozo et al. 1997) |
| Ontario, Canada | Mixed deciduous | 324 | 98 | (Oelbermann and Gordon 2000) |
| Idaho, U.S. | Mixed deciduous/coniferous | 25-414 | 86-100 | (Minshall et al. 1992) |
| North Carolina, U.S. | Mixed deciduous | 625-714 | 69-80 | (Wallace et al. 1995) |
| North Venezuela | Cloud forest | 532 | 92 | (Cressa and Weibezahn 1976) |
| NSW, Australia | Eucalyptus forest | 678 | 65 | (Campbell et al. 1992) |

## GENERAL PATTERNS AND MECHANISMS OF LITTER DECOMPOSITION IN STREAMS

Decomposition processes have been largely studied in the last four decades (Tank et al. 2010), and consequently, an extensive theoretical framework has been developed. Decomposition is a complex process involving three main mechanisms, which are overlapping over time: leaching, microbial conditioning and fragmentation (Petersen and Cummins 1974, Webster and Benfield 1986, Gessner et al. 1999, Allan and Castillo 2007).

### Leaching

Leaching is defined as the loss of soluble compounds from the leaf, such as phenolics, carbohydrates and amino acids (Bärlocher 2005a). This mechanism dominates at the early stages of leaf litter decomposition (fallen leaves just immersed in water) and results in a rapid mass loss in the first week, with a peak during the first 24 to 48 hours (Bärlocher 2005a), although the leaching of some compounds can take longer (France et al. 1997). Leaf mass leaching might account for up to 42% of the initial mass (Abelho 2009), but it greatly depends on leaf species (Maloney and Lamberty 1995, Taylor and Bärlocher 1996). For instance, in a Mediterranean stream, Casas and Gessner (1999) found that *Populus nigra* and *Salix atrocinerea* leached 25%, *Rubus ulmifolius* 15% and *Platanus orientalis* 10% of their initial mass in the first 3 days. Environmental factors such as temperature and turbulence might also affect the rate of leaching (Ardón and Pringle 2008). Drying of leaves might increase their

ulterior leaching (Gessner 1991, Bärlocher 1992b), although this process can vary even between trees of the same species (Taylor and Bärlocher 1996).

**Conditioning**

Recently-shed leaves of many tree species are of low palatability and have low nutritional value for stream invertebrates, and thus, are hardly consumed unless they are first conditioned by microbes (mainly bacteria and fungi) (Petersen and Cummins 1974, Gessner et al. 1999, Allan and Castillo 2007). During the conditioning phase, the chemical composition of plant tissue is modified mainly through four mechanisms: 1) conversion of plant tissue into microbial materials (microbial growth); 2) breaking of complex leaf molecules into simpler ones by means of microbial extracellular enzymes; 3) mechanical alteration of leaves mainly due to fungal hyphae growing; and 4) microbial nutrient incorporation, mainly inorganic nitrogen and phosphorus dissolved in the stream water (Bärlocher and Kendrick 1975, Gessner et al. 1999). Indeed, although conditioning conceptually remarks the relationship between microbes and shredders, this phase mainly involves the microbial decomposition processes (Gessner et al. 1999), which have been broadly studied (e.g. Gessner and Chauvet 1994, Sridhar and Bärlocher 2000, Gulis and Suberkropp 2003a, Duarte et al. 2010).

Microbial decomposer activity is considered one of the most important mechanisms of leaf litter breakdown (Gessner and Chauvet 1994). Notwithstanding, microbial contribution and colonisation can be very variable depending on leaf species, stream environmental variables, and characteristics of the microbial colonizing species (Abelho 2001). On the other hand, fallen leaves can also be a substrate for autotrophic communities associated with biofilm formation, but, due to the ephemeral condition and apparently low contribution to the detritus pool, the influence of primary producers in organic matter dynamics has been largely unexplored (Golladay and Sinsabaugh 1991). Nevertheless, recent studies remark a possible stimulation effect of algae on litter decomposition (Danger et al. 2013, Kuehn et al. 2014).

**Fragmentation**

Fragmentation results in the release of fine-particulate organic matter to the stream water either from shredding, consumption and production of faeces by invertebrates, or from physical fragmentation due to water abrasion (Gessner et al. 1999, Abelho 2001, Ferreira et al. 2006). Some shredding aquatic insects and crustaceans are the most common consumers of CPOM, principally due to specialized mouthparts, and flora and enzymes present in their digestive tracts (Bärlocher and Porter 1986, Allan and Castillo 2007). They use leaf litter as a food resource and

incorporate leaf material into secondary production (Graça and Canhoto 2006, Canhoto and Graça 2008). Shredders seem to feed preferentially on conditioned leaf litter (Graça et al. 2001a). Therefore, the relevance of biological fragmentation can be modulated indirectly by factors limiting microbial colonisation (explained above) and directly by factors affecting shredders (Connolly and Pearson 2013), such as spatial and temporal variations in CPOM standing stocks (Graça 2001). Moreover, shredder species might vary in their ability to digest leaf litter, which appears to be influenced by their feeding behaviour, digestive physiology, the range of palatability of their food resources and their capacity to overcome defence mechanisms of leaf-colonizing microorganisms (Barlocher 1985).

Physical fragmentation mechanisms are still poorly understood, their relevance on litter breakdown increase with high flow and current velocity, but they also depend on leaf resistance, which varies between leaf species (Abelho 2001)

Litter breakdown is, thus, an integrative process that includes physical, microbial and invertebrate mechanisms (Fig. 1). The process recycles nutrients and results in products such as FPOM and DOM, soluble inorganic compounds, $CO_2$, microbial and invertebrate biomass (Baldy and Gessner 1997, Gessner et al. 1999, Hieber and Gessner 2002).

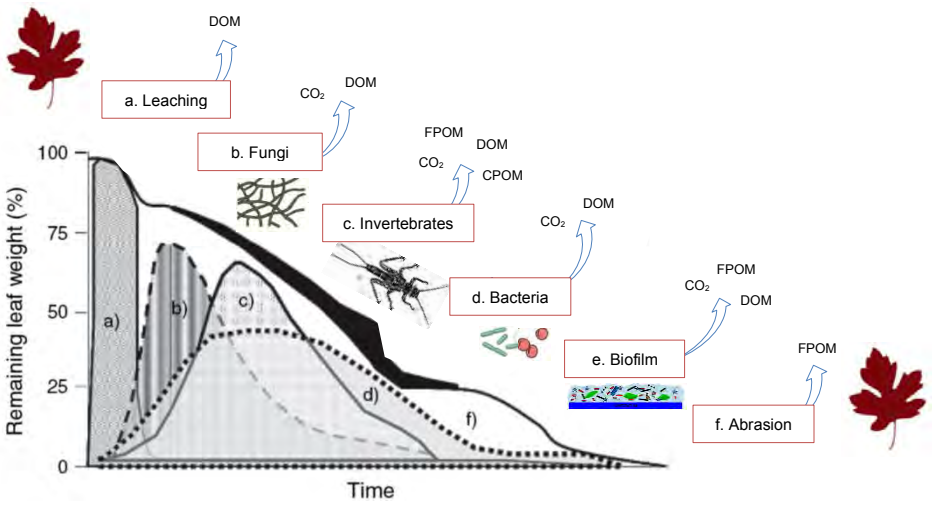



Figure 1. Mechanisms, products and biological communities involved in litter processing in streams. Modified from (Wantzen et al. 2008).

## FACTORS CONTROLLING LITTER DECOMPOSITION IN STREAMS

Leaves are decomposed at different rates (Petersen and Cummins 1974), depending on both internal and external factors (Webster and Benfield 1986). Internal factors are mainly referred to leaf chemical and physical characteristics, including the

content of nutrients (particularly N and P) and unpalatable substances such as lignin, the presence of chemical inhibitors, and cuticle toughness (Meentemeyer 1978, Ostrofsky 1997, Cornwell et al. 2008). Quality of leaves can significantly vary due to several reasons, such as taxonomic differences (Ostrofsky 1997), within-species genetic variations (LeRoy et al. 2007), phenological status (Kochi and Yanai 2006) or tree condition (Lecerf and Chauvet 2008a). The physico-chemical characteristics of leaves determine decomposer colonisation and activity (e.g. Dang et al. 2007) and detritivore consumption rates (e.g. Kominoski and Pringle 2009). Generally, litter breakdown rates are slower for leaves with high lignin levels, low nitrogen or phosphorus concentration or higher toughness (e.g. Triska and Sedelll 1976, Gessner and Chauvet 1994, Martínez et al. 2013).

External factors are mainly related to environmental variables such as temperature, dissolved nutrients, pH, oxygen concentration, physical abrasion and hydromorphological parameters (Reice 1974, Webster and Benfield 1986, Young et al. 2008, Tank et al. 2010). There are plenty of studies assessing the influence of environmental conditions on litter breakdown; however, the effects are not easy to disentangle given the concomitant variation in other variables and the particular responses of decomposers and detritivore communities (Webster and Benfield 1986, Suberkropp and Chauvet 1995, Friberg et al. 2009). However, certain tendencies can be described regarding extrinsic environmental factors. For example, temperature directly regulates metabolic rates, and consequently, plays an essential role in leaf processing (Dang et al. 2009, Geraldes et al. 2012, Friberg et al. 2013). Phosphorus and nitrogen concentrations promote litter breakdown, but only until a threshold beyond which breakdown rates decrease again (Woodward et al. 2012). On the other hand, low pH and oxygen concentrations can retard, and even inhibit litter breakdown (Pascoal et al. 2003, Medeiros et al. 2009, Simon et al. 2009). Although less studied, hydromorphological parameters, such as flow and bed substrata also influence leaf litter breakdown in streams due to their effect on biological communities (Reice 1974, Lytle and Poff 2004, Ferreira et al. 2006, Flores et al. 2013).

## MICROBIAL DECOMPOSERS AND EXTRACELLULAR ENZYME ACTIVITIES

### Fungal and bacterial communities in leaf litter

Taxa from several fungal phyla such as Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota, as well as bacteria and archeobacteria are able to colonize leaf litter (Nikolcheva and Bärlocher 2004, Manerkar et al. 2008). Despite the high diversity of microbial decomposers, most studies in microbial breakdown have

focused on aquatic hyphomycetes (Ascomycota), as they produce conidia that are easily identifiable microscopically to the species level. Nevertheless, species with high sporulation rates are not always the ones that produce more biomass (Duarte et al. 2006), and it is the active mycelium, not the conidia, which decomposes the leaves. Recently, molecular techniques allow the detection of sporulating as well as non-sporulating species (Bärlocher 2010). Additionally, molecular techniques allow, at least semi-quantitatively, to estimate the relative biomass of the fungal community (Nikolcheva and Bärlocher 2005).

Leaf colonisation starts before they reach the stream, but rapidly (in the first week) aquatic fungal species arrive and replace terrestrial ones (Nikolcheva et al. 2005). Fungal assemblages can change through the decomposition process, and although some species have been classified as early and late colonizers (Gessner et al. 1993, Nikolcheva et al. 2005, Duarte et al. 2010), there is not a clear-cut replacement of one group of fungi by another (Bärlocher 1992a). Bacterial species also might show successional changes through leaf decomposition (Duarte et al. 2010); however, the studies addressing diversity of leaf-associated bacteria in streams are scarce and mostly based on cell morphology (e.g. Hieber and Gessner 2002) or on cultivable taxa (Suberkropp and Klug 1976). Studies addressing microbial decomposition are generally based on measurements of fungal and bacterial biomass (Suberkropp 2001, Hieber and Gessner 2002, Gulis and Suberkropp 2003b, Duarte et al. 2009) or productivity (Suberkropp and Weyers 1996, Baldy et al. 2002, Pascoal and Cássio 2004), as well as on fungal sporulation rate (Suberkropp 2001, Graça et al. 2002, Pérez et al. 2012). Generally, it has been observed that fungi quickly grow and produce conidia after arriving to litter, and fungal production and sporulation rates peak in the first two weeks and then rapidly decrease (Bärlocher 2009). Fungal biomass (measured as ergosterol or ATP) typically lags behind production and sporulation, and the subsequent decline is more gradual (Gessner et al. 1999, Bärlocher 2009). Bacterial colonisation can lag behind mycelia development (Gessner et al. 1999); however, sometimes no changes are detected, or even an increase is shown in the final stages of decomposition (Baldy et al. 2002, Duarte et al. 2010, Artigas et al. 2011).

**Role of extracellular enzymes**

Fungi and bacteria play a key role in leaf litter decomposition mainly thanks to their extracellular enzyme capabilities (Chamier 1985, Zemek et al. 1985, Moorhead and Sinsabaugh 2000). Microbes produce cellulases, hemicellulases, pectinases and phenol oxidases to degrade carbon-rich structural compounds, such as lignocelluloses,

which are resistant to degradation (Fig. 2); while peptidases, ureases and phosphatases, allow microbes to uptake inorganic nutrients, and thus, compensate the low N and P content of leaf litter (Chamier 1985, Sinsabaugh et al. 2002b, Moore et al. 2004, Romaní et al. 2006a).

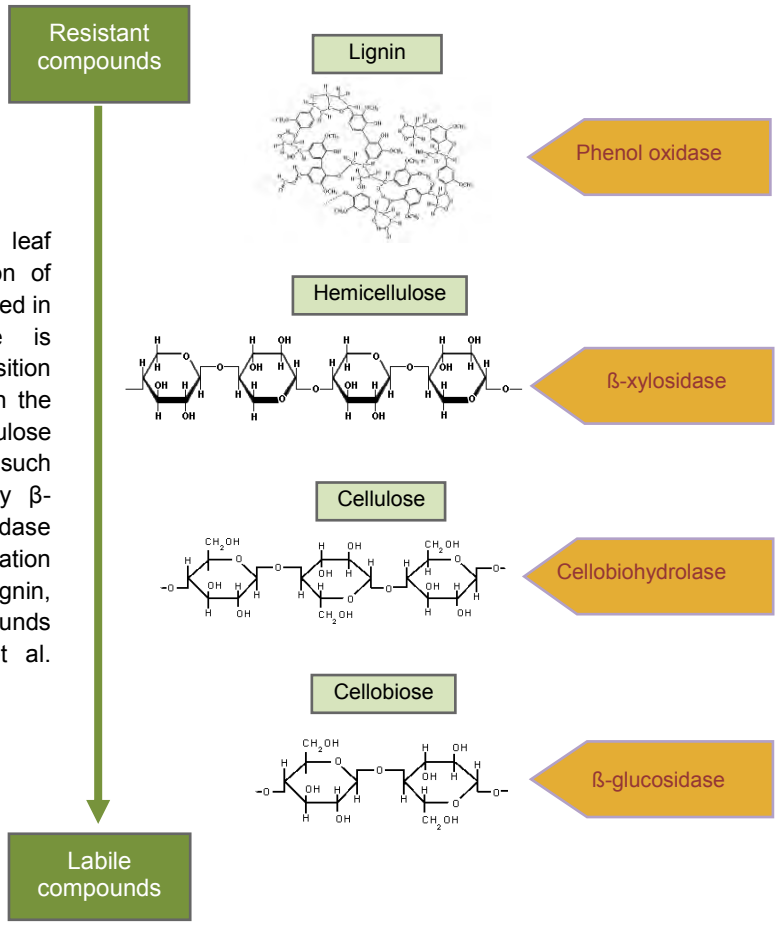



Figure 2. Relationship between leaf litter compounds and the action of extracellular enzyme activities used in this study. Cellobiohydrolase is involved in cellulose decomposition while β-xylosidase is involved in the last step of hemicellulose decomposition. Disaccharides such as cellobiose are degraded by β-glucosidase. Finally, phenol oxidase activity is related to the degradation of resistant compounds like lignin, phenolic and humic compounds (Chamier 1985, Sinsabaugh et al. 2002a).

Changes in litter composition trigger different enzyme activities throughout decomposition. Thus, β-glucosidase might be especially active during early stages of decomposition thanks to labile compounds releasing by leaching, but it is also activated after cellulose degradation. Phenol oxidase tends to be active over all the leaf litter decay process but increases its activity with lignin content, whereas β-xylosidase also might depend of previous enzyme steps in the degradation of hemicellulose (Sinsabaugh et al. 2002a, Fig. 2). In addition, changes in community assemblages also might affect enzyme activity, since fungal species differ in their abilities to produce decomposing enzymes (Suberkropp and Klug 1980). Overall, the expression of extracellular enzyme activities has been directly related to leaf mass loss in streams and soil (Sinsabaugh and Linkins 1993, Simon et al. 2009), although a detailed analysis of these enzyme capabilities in stream leaf litter decomposition is still lacking.

**Fungal-bacterial interactions**

Fungi and bacteria live in close proximity to each other, utilising leaves as a carbon source. However, they differ in the manner in which they access leaves. Fungi are able to penetrate leaves with their hyphae, whereas bacteria attach to the external surfaces or colonise inside the leaves in association with hyphae growth (Baschien et al. 2009). Therefore bacteria and fungi differ in their function, and are expected to interact in different ways during litter processing.

Fungi are considered to be the responsible for microbial litter decomposition (Hieber and Gessner 2002, Pascoal and Cássio 2004), although bacteria can play an important role, mainly in the late stages of decomposition (Duarte et al. 2010, Artigas et al. 2011), and in some environments such as freshwater marshes (Buesing and Gessner 2006). Some authors have also suggested that bacteria and fungi play complementary roles through their particular enzyme capabilities, being fungi able to decompose complex molecules such as lignin and hemicellulose, whereas bacteria are efficient in the decomposition of more simple molecules (i.e. simple polysaccharides, Romaní et al. 2006a).

Several studies have shown a constant antagonist interaction between bacteria and fungi. Fungal species are capable to inhibit bacterial growth (Gulis and Suberkropp 2003b, Mille-Lindblom and Tranvik 2003) and in the presence of bacteria fungal biomass, fungal sporulation and some enzyme activities might be reduced (Wohl and McArthur 2001, Gulis and Suberkropp 2003b, Romaní et al. 2006a, Baschien et al. 2009). Antagonistic interactions have been explained by direct mechanism such as competition for substrata or nutrients, and by indirect mechanisms through extracellular compounds. By contrast, positive interactions between bacteria and fungi have been also reported. Romaní et al. (2006a) found that bacteria grow better together with fungi than alone, apparently because fungi provides bacteria with resources they cannot acquire on their own. Nevertheless, other studies have failed to detect bacterial-fungal interaction (Das et al. 2012). This suggests that fungal-bacterial interaction may be dependent on microbial traits and environmental conditions.

**Microbes-invertebrate interactions**

A simple approach to evaluate the interaction between microbial decomposers and invertebrate detritivores is to compare the microbial performance on leaves enclosed in fine mesh, which preclude shredder access to leaves, with that of leaves placed in coarse mesh, where shredders can access (Ferreira and Graça 2006). Both synergistic and antagonistic interactions have been observed (Canhoto and Graça 2008). Microbes are responsible for leaves conditioning and increase their nutrient

contents, thus favouring invertebrate consumption (Bärlocher and Kendrick 1975, Foucreau et al. 2013), and microbial enzymes may be used inside insect guts (Canhoto and Graça 2008). On the other hand, invertebrate feeding can disrupt physical barriers of leaves, enhance their surface-to-volume ratio, and increase basal resources by egestion and excretion, favouring microbial performance (Sabetta et al. 2000, Canhoto and Graça 2008, Villanueva et al. 2012). Moreover, protists can modulate the activity of surface-associated microbial communities by grazing bacteria (Risse-Buhl et al. 2012). On the other hand, invertebrates and microbes can compete for food resources (Bärlocher 1980). Invertebrate also predate bacterial cells, mycelia or fungal spores (Suberkropp et al. 1983, Suberkropp and Wallace 1992, Hahn and Höfle 2001).

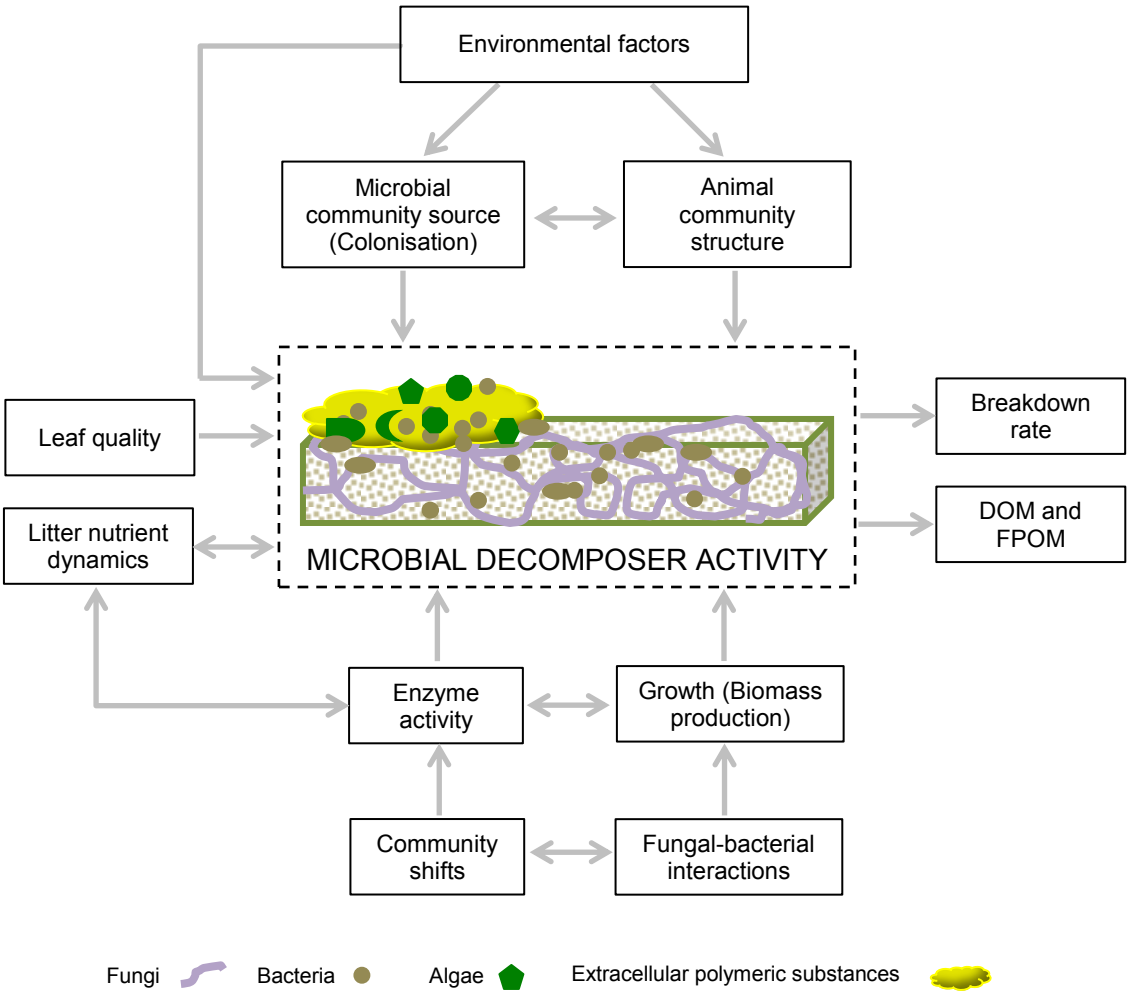



Figure 3. Scheme of microbial mediated litter decomposition. Modified from Gessner et al. (2007).

Overall, the leaf litter breakdown process and specifically the microbial decomposition can be summarized from a decomposer point of view (Gessner et al. 2007, Fig. 3). Therefore microbial decomposition will depend on the diverse factors

explained through this section, such as the characteristics of the source community present in a stream, their enzyme activity capacities, the biotic interactions, and of course, the factors that governs total breakdown process (environmental factors and litter quality, Fig. 3).

## MEDITERRANEAN INTERMITTENT STREAMS AND EFFECTS OF DROUGHT

### Mediterranean streams

Breakdown rates and the mechanisms involved in litter processing are highly dependent on the stream environment and are modulated by microbial and invertebrate responses to environmental variations (Webster and Benfield 1986), as discussed in the previous section (see “Factors controlling litter decomposition in streams”). Temperature, pH, nutrient concentrations, oxygen level, and flow are the principal controlling factors of biotic activity in freshwater ecosystems and can vary among sites (Allan and Castillo 2007), but also in systems exposed to strong seasonality as Mediterranean streams. This suggests that leaf litter decomposition in Mediterranean streams will show seasonal changes.

Mediterranean-climate regions include areas surrounding the Mediterranean Sea, parts of western North America, parts of West and South Australia, South-Western South Africa and parts of central Chile. They are characterized by predictable seasonal variation in precipitation and temperature, with hot, dry summers and cool, wet winters (Gasith and Resh 1999). In addition, the total rainfall varies considerably between years, and in some areas water may be stored in subterranean aquifers, which may moderate seasonal fluctuations. Therefore, in wet Mediterranean regions, streams usually maintain permanent flow or at least hold surface water throughout the year, but in drier Mediterranean zones, streams are often intermittent with summer drought periods (Gasith and Resh 1999).

Biota living in intermittent streams exposed to Mediterranean climate regime has to deal with extreme temperatures and flow variability, from drought to severe floods, showing a marked seasonality in abundance and community composition (Gasith and Resh 1999). Furthermore, drought in streams determines complex hydrological dynamics including flow gradients, and longitudinal connections and disconnections (Larned et al. 2010), which structures biological communities and influence nutrient cycles and organic matter processing (Ylla et al. 2010, Datry et al. 2011, von Schiller et al. 2011, Resh et al. 2012).

**Drought and leaf litter breakdown in intermittent streams**

In intermittent streams, water stress of riparian vegetation leads to early and prolonged leaf abscission periods (Gasith and Resh 1999). For example, in the Fuirosos stream, Acuña et al. (2007) found litter fall to extend from April to December. Therefore, litter in the stream is potentially exposed to summer drought and affected by the varied conditions observed through the drying process and the flow resumption.

Drought starts with flow reduction that breaks hydrological connectivity and reduces hydraulic heterogeneity. As drought proceeds, the watercourse is disconnected and the aquatic habitat suffers an abrupt decline, being reduced to pools of different sizes. Pools are normally ephemeral and rapidly water quality can change, concentrating the biota in a reduced harsh habitat. In isolated pools, oxygen concentration and pH tend to decrease, whereas dissolved humic substances, conductivity and temperature increase (e.g. Acuña et al. 2005, Ylla et al. 2010). If the drought conditions continue, pools eventually get dry and the streambed becomes a terrestrial corridor (Fig. 4). Depending on the stream, the period of total drying can last from days to months, and then, when rainfall returns, flow is resumed and the aquatic ecosystem is recovered. Depending on the local hydrological regime, the flow in a particular reach can be connected or disconnected during a summer drought period, and flow recovering might be abrupt or gradual (Humphries and Baldwin 2003, Lake 2003, Steward et al. 2012).

Since the drought process is a complex gradient of conditions between terrestrial and aquatic phases, processing of leaf litter during this period can be affected in several ways. It can be directly affected via abiotic processes such as photo-degradation during terrestrial phase (Austin and Vivanco 2006, Gallo et al. 2006, Dieter et al. 2011), and/or indirectly by decomposer and detritivore community sensitivity to pool formation, emersion-immersion cycles, floods associated with the flow resumption, and historical regimes in drought frequency and intensity in a particular reach (Fig. 4). Relatively few studies have assessed litter breakdown responses to drought, some of them have compared permanent with intermittent streams and have shown leaf litter breakdown tend to be lower in temporary streams, related to lower shredders abundances and slow recovering of invertebrates after flow resumption (Hill et al. 1988, Richardson 1990). Other studies have evaluated the influence of frequency and intensity of drought in litter decomposition during the wet period (Richardson 1990, Pinna and Basset 2004, Pinna et al. 2004, Datry et al. 2011). These studies observed that litter processing in intermittent streams can be decelerated due to drought and this was mainly related to the sensitivity of invertebrate to long and frequent drought events. Other studies have tested the impact of emersion and immersion on

decomposing leaves, finding breakdown rates being apparently reduced by the accumulative time which the leaves were exposed to drought conditions, as well as microbial biomass and activity are greater during immersion (Maamri et al. 1997, 1998, 1999, 2001, Corti et al. 2011, Bruder et al. 2011). These studies also observed low resistance of invertebrate to emersion and deceleration of microbial processes during the terrestrial phase, although microbial activity seemed to be rapidly recovered after immersion. In addition, harsh condition found in pools can reduce microbial activity, shredder colonisation and leaf breakdown rates (Schlief and Mutz 2011). Recent studies have experimentally found leaf litter quality might be changed by UV-radiation and anoxic conditions in pools, which in turn can affect breakdown rates and microbial communities once the flow is recovered, although this effect was variable among leaf species (Dieter et al. 2011, 2013). Despite the existent knowledge about drought effect on leaf litter breakdown process, further research is necessary to contrast the general patterns observed so far, as well as to answer several aspects that are still unclear, such as the microbial enzyme response to drought, interactions and role of terrestrial and aquatic decomposer communities, resistance and resilience of microbial communities to drought conditions, and interactive effects with other disturbance such as anthropogenic nutrient enrichment.

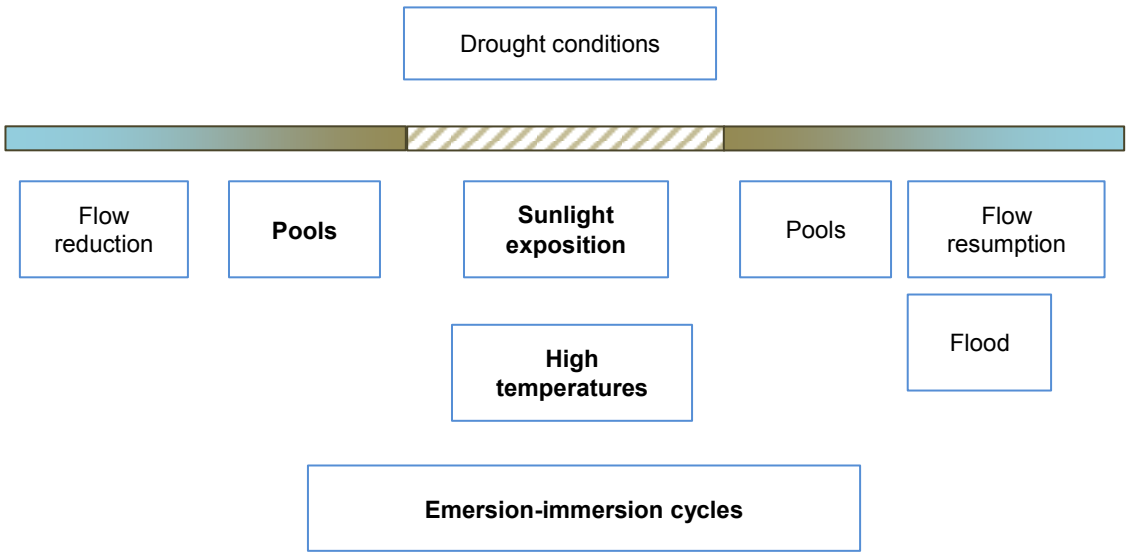



Figure 4. Potential conditions of drought that can affect leaf litter breakdown in streams. The drought consequences studied in this thesis are in bold

### Global change and drought

Human activities are greatly altering the climate worldwide and affecting biodiversity and ecosystem processes. Freshwaters are amongst the most affected due to the demand for resources, which are conducting to the deterioration and loss of

ecosystem services. Although there are several threats to rivers, climate change is probably one of the biggest concerns and the one to which ecologists are devoting more attention (Allan and Castillo 2007).

In a context of climate change, drier climatic conditions are forecasted in some temperate regions (Beniston et al. 2007, IPCC 2013). Increased air temperatures, scarce precipitations and intensive human water usage (e.g. irrigation, drinking water) might determine changes in hydrological trends of aquatic ecosystems, increasing frequency, intensity and duration of extreme events such as droughts (Meyer et al. 1999, Andersen et al. 2006). Moreover, combined effects of land-use patterns and climate change can affect the spatial and temporal extent of intermittent streams, even converting permanent streams in temporaries (Palmer et al. 2008, Acuña et al. 2014).

Therefore, under global change trends, streams from temperate wetter regions could be exposed to drought, for which biological communities are not evolutionally adapted, threatening the ecological integrity of these aquatic ecosystems (e.g. Schlief and Mutz 2011). Moreover, longer and extreme drought periods expected for natural intermittent streams also might have important consequences for total carbon budget, reducing stream efficiency to recycling nutrients related with slower breakdown rates (Datry et al. 2011).

Knowledge of functioning of natural intermittent streams could be applied to other streams at risk of temporality (Larned et al. 2010). However, as was explained above, there are still many aspects to be resolved regarding to leaf litter breakdown mechanisms under drought disturbance, and studies assessing this topic in permanent streams are scarce. This thesis wants to contribute to fill this lacks and give tools to better understanding litter breakdown process in Mediterranean intermittent streams.

## OBJECTIVES OF THIS STUDY

The main goal of this study was to elucidate the principal controlling factors and microbial mechanisms involved in litter processing in Mediterranean intermittent streams. It is expected that litter breakdown will be affected by temperature and flow fluctuations, as well as by environmental conditions associated with summer drought period, which are becoming more extreme under current global change scenario in Mediterranean streams. Therefore seasonal variation, microbial dynamics and summer drought impact were assessed in order to understand the potential consequences of global change predictions in this kind of ecosystems.

Additionally, we tested the drought disturbance, focusing on the emersion effect on decomposing leaves, in temperate permanent streams non-naturally exposed to

drought conditions, and evaluated the potential interaction effect of drought and nutrient enrichment in these systems.

The specific objectives of the thesis are the following:

1. To identify the primary factors driving leaf breakdown in a Mediterranean stream subjected to strong seasonal variations and to investigate the relative contribution of microbes and invertebrates to total breakdown seasonality. It is expected that the relative role of biological components will vary throughout the year according to a differential sensitivity to temperature, flow and water quality variations, as well as particular seasonality of biota. It is also expected that temperature will drive seasonality in litter breakdown, excepting during the drought phase, when other factors such as flow, oxygen, and/or pH would take a key role as drivers of the breakdown process (Chapter I)
2. To determine the fungal and bacterial dynamics and microbial mechanisms involved in the leaf litter decomposition process in an intermittent Mediterranean stream; exploring whether seasonal environmental variation and invertebrate affect enzyme activity and microbial assemblages. It is expected that the dynamics of the microbial assemblages and enzyme mechanisms through leaf decomposition will be modulated by seasonal changes and/or presence of invertebrates. (Chapter II)
3. To evaluate the effect of a long summer drought phase on litter processing in an intermittent Mediterranean stream. It is hypothesised that litter quality will change with drought exposition, and will affect breakdown when immersing the leaves in the stream once the flow is recovered. Additionally, it is expected that the drought effect will influence the composition and performance of microbial decomposers and invertebrate detritivores colonizing the leaves once the flow is recovered. (Chapter III)
4. To evaluate the microbial decomposer response to drought in a permanent “pristine” stream, specifically assessing whether different lengths of emersion period is affecting microbial litter processing. It is hypothesised that longer exposition to emersion conditions will determine higher functional and structural microbial community changes. (Chapter IV)
5. To evaluated the potential interactive effect of emersion of decomposing leaves and nutrient enrichment in permanent temperate streams. It is expected that emersion exposure will affect both kind of systems, but streams with different

trophic status are inhabited by different microbial decomposer communities, thus different responses to emersion exposition are expected. (Chapter V)

# Materials and Methods

STUDY SITES

To achieve the objectives of this thesis, several sampling and experiments were conducted in three different low order streams: one intermittent Mediterranean stream experiencing low anthropogenic impact, and two permanent Atlantic streams: one oligotrophic and one eutrophic (Fig. 1).

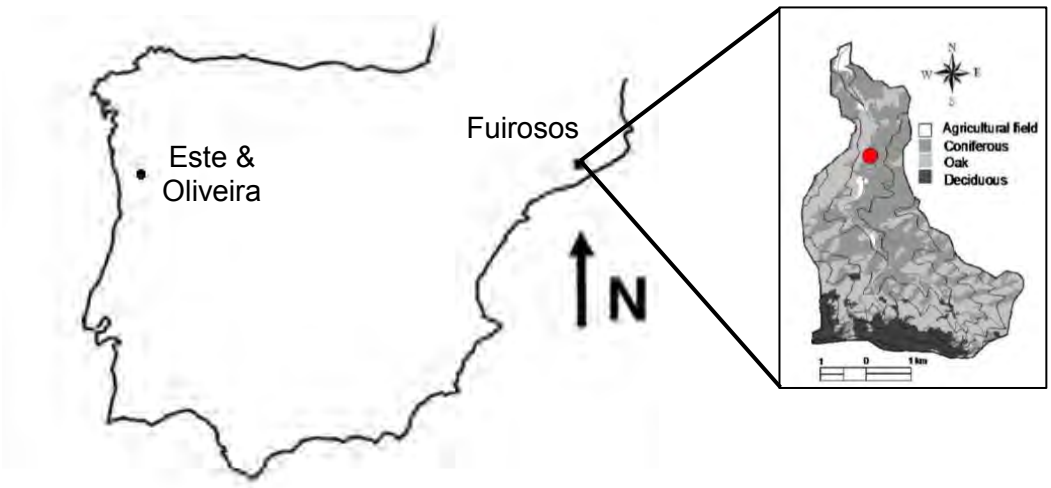



Figure 1. Geographical location of study sites in the Iberian Peninsula. Este and Oliveira are Atlantic temperate streams and Fuirosos is a Mediterranean stream.

THE FUIROSOS: A MEDITERRANEAN INTERMITTENT STREAM

The Mediterranean intermittent stream (Fuirosos) is a third-order stream located in Catalonia, NE Spain (latitude 41º42' N, longitude 2º34'W, 50–770 m.a.s.l.). The Fuirosos drains a small granitic catchment of approximately 15.6 $km^2$. This stream is under a Mediterranean climate regime, which is characterized by high inter and intra annual variation in air temperature (ranging from 4 ºC to 28 ºC) and rainfall (ranging from 200 mm to more than 1000 mm per year, primarily recorded in autumn and spring but with occasional summer storms) (Romaní et al. 2006b, Sabater et al. 2008, Sanpera-Calbet 2014); and accordingly high water temperature and flow variation in an annual cycle (Fig. 2). The catchment is located in a protected natural area (the Montnegre-Corredor Natural Park) mostly covered by forest (90 % of total area). The catchment was traditionally used for forestry, pasture and agriculture, and although it had been gradually abandoned during the last 50 years, pasture is acquiring relevance recently (Vazquez et al. 2013, Sanpera-Calbet 2014).

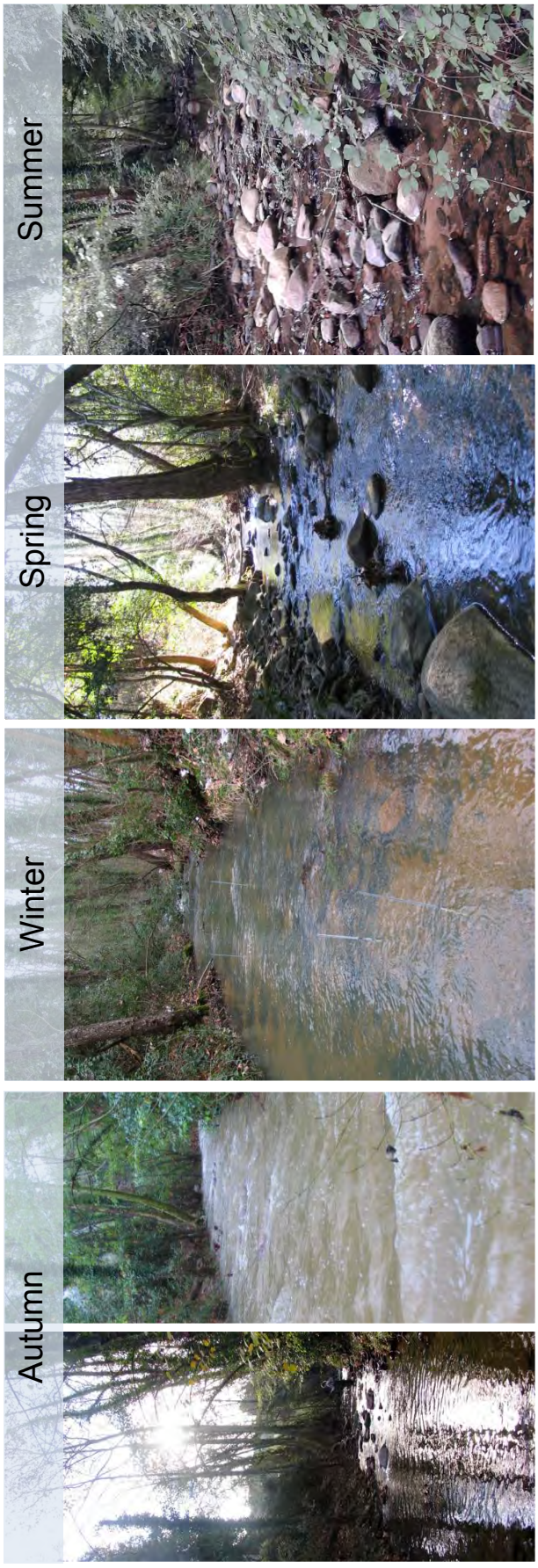



Figure 2. Different views of the Fuirosos stream showing the intense flow variations throughout the hydrological cycle, from autumn to summer.

The present study was developed in the middle part of the stream in a reach of 5-7 m wide and 100 m in length. In this section of the stream, mean flow is around 7–20 L $s^{-1}$ and discharge is intermittent hence the stream typically dries up in summer. The summer drought period is of variable duration (1-3 months) and variable spatial extent among years (Vazquez et al. 2013). In general, nutrient concentrations are low throughout the year and it is considered that biological activity is potentially limited by phosphorus for most of the hydrological cycle (Sabater et al. 2005, von Schiller et al. 2011).

The riparian vegetation is well developed, and mainly composed by plane (*Platanus Acerifolia* [Aiton] Willd.), alder (*Alnus glutinosa* [L.] Gaertn.), poplar (*Populus nigra* L.) and hazelnut (*Corylus avellana* L.). Riparian inputs is in average 912 g dry mass $m^{-2}$ $y^{-1}$ and annual distribution of litter inputs is strongly determined by the summer drought, presenting bimodal annual cycles with a higher summer peak (caused by hydric stress) in years with intense summer drought followed by the autumn peak, but unimodal pattern with only the autumn peak in years without drought (Sanpera-Calbet 2014). DOC concentration in stream water ranges from 2 to 3 $mgL^{-1}$ under basal discharge conditions, but during the stream recharge period (September–October) stream DOC concentrations increase to 10–15 mg L–1, occasionally reaching 20 mg $L^{-1}$, especially after an intense summer drought period (Bernal et al. 2002, Vázquez et al. 2007) .

THE OLIVEIRA AND THE ESTE: PERMANENT ATLANTIC STREAMS

Some experiments of this thesis were conducted in two permanent Atlantic streams belonging to the Ave River basin, located in the Northwest of Portugal: the Oliveira stream (latitude 41.58º63'' N, longitude -8.22º51''W, 232 m.a.s.l.) with low human pressure and the Este river (latitude 41.52º70'' N, longitude -8.43º51''W, 148 m.a.s.l.) highly impacted by human activities (Fig. 3).


Figure 3. View of the studied sites of the permanent Atlantic streams in Northwest of Portugal: the oligotrophic stream Oliveira (a) and the eutrophic stream Este (b).

The Oliveira is a fourth-order stream and its riparian vegetation is dominated by *Alnus glutinosa* (L.) Gaertn., *Quercus robur* L., *Castanea sativa* Mill. and *Populus nigra* L. The stream bedrock is composed by boulders and pebbles (Geraldes 2011). The studied reach is about 1.5 m deep, 10 m wide and 20 m in length.

The Este, a third-order river, flows through an agricultural area with high population density and then through the town of Braga and its industrial park. The selected site was located before the industrial park and presents high concentration of nutrients. The studied reach is about 2 m deep, 7 m wide and 20 m in length and its bottom consists mainly of gravel and mud. The riparian vegetation is dominated by *Alnus glutinosa* (L.) Gaertn., *Salix sp.*, *Populus nigra* L., *Quercus robur L.*, and *Rubus ulmifolius* Schott (Duarte et al. 2008, 2009).

PHYSICAL AND CHEMICAL PARAMETERS OF STREAM WATER

In all the experiments carried out in this thesis, pH, current velocity, conductivity, and oxygen were recorded in the field with specific probes (Hach multiprobe meter in the intermittent stream and Multiline 340i; WTW, Weilheim, Germany in the permanent streams). Measures were performed for each point in which litterbags were immersed. Temperature was measured with data loggers submerged in the same reach where each experiment was conducted (SmartButton, ACR System Inc. in the intermittent stream and Hobo Pendant UA-001-08, Onset Computer Corp., Massachusetts, USA in the permanent streams). Continuous temperature measurements with 10 minutes (intermittent stream) or 15 minutes (permanent streams) time step were recorded and in the necessary cases (Chapters I and V) values were used to calculate breakdown rates in degree-days.

Additionally, in the Fuirosos stream, continuous discharge values throughout the study periods were provided by Andrea Butturini and Eusebi Vázquez from the University of Barcelona. The discharge was calculated from a regression between discharge calculations (measured by mass balance calculations using the “slug” chloride addition method) and water level measured continuously by a water pressure transducer (Vázquez et al. 2007).

On each sampling date water was collected, transported to the laboratory in a cool box, and filtered (Nylon filters, 0.2 µm pore size) to analyse nutrient concentrations. In the Fuirosos stream water was collected in triplicate and preserved at -20 ºC until forward processing. Nitrate was analysed by ion chromatography (761 IC, Metrohm, Switzerland), and soluble reactive phosphorus by the ascorbic acid method (APHA 1989). In the permanent streams the collected water was analysed for

nutrient concentrations within 24 h after sampling, and quantified with a Hach DR/2000 photometer (Hach company, Loveland, CO) using the specific methods and programs for the measurement of nitrates (cadmium reduction program), nitrites (diazotization method), ammonium (salycilate method), and soluble reactive phosphorus (ascorbic acid method).

LITTERBAG TECHNIQUE

Leaf-litter decomposition experiments were performed with *Populus nigra* L. leaves in each studied stream using the litterbag approach described in Graça et al. (2005). Recently fallen poplar leaves were collected in October 2009 under four contiguous trees at the Fuirosos catchment and in October-November 2010 under trees located near to University of Girona. Leaves were air-dried for 15 days, and stored in the dark in cardboard boxes until each experiment performance. Different bag types were used according to the aim of each study conducted: coarse bags that allow the evaluation of total breakdown process, and fine bags that avoid invertebrate access (Table 1).

For each litterbag experiment, three grams (± 0.1 g) of air-dried leaves were enclosed in each bag and submerged in the stream. In order to avoid the loss of the litter bags, in the intermittent stream bags were tied to a rope, which was crossing the stream (the rope was tied to the surrounding trees or rocks at the stream edges); and in the permanent streams bags were directly tied to surrounding trees or rocks, and they had litter weights to facilitate sink. Periodical retrieval of the litterbags was performed regarding each particular experimental design.

Table 1. Types of litterbags and mesh sizes used in each experiment according to the aim of each specific study.

| Chapter | Aim | Coarse mesh size bag (mm) | Fine mesh size bag (mm) |
|---|---|---|---|
| I | Microbial and invertebrate contribution to litter breakdown | 10 | 0.2 |
| II | Invertebrate influence on microbial processes | 10 | 0.2 |
| III | Drought effect on total breakdown process | 10 | |
| IV | Drought effect on total breakdown process | 5 | |
| V | Drought effect on total breakdown process | 5 | |

Bags retrieved from the stream were opened in the field or in the laboratory. Leaf material remaining was gently sorted and rinsed with stream water to remove invertebrates, exogenous organic matter and sediments. Litter disks of ~1.1 cm-diameter were cut from each litter bag using a core borer and placed in vials for measuring bacterial biomass (~2 disks), fungal biomass (~5 disks), fungal sporulation

(~10 disks), extracellular enzyme activities (1 or 2 disks for each measured enzyme), microbial assemblages (~10 disks) and leaf dry mass of disks (~10 disks). Leaf material was also collected for leaf composition: C and N content (2 to 4 mg per sample), P content (2 to 4 mg per sample), and total fibber, lignin and cellulose content (~200 mg per sample). The total amount of disks, specific parameters considered and sampling strategy varied among the studies conducted in the thesis (see details in each specific chapter). The plant material remaining in bags was then placed in aluminium foil, oven-dried (48 h, 60 ºC) and weighed (precision 0.001 mg; microbalance Sartorius 2MP), to calculate dry mass. For each litterbag experiment, a set of four extra bags was prepared to measure the initial dry mass and litter mass remaining by bag was calculated based on initial corrected dry mass (Graça et al. 2005). In the chapter I and II, the oven-dried leaf samples were ashed (4h, 500 °C), and weighed again to calculate AFDW (ash free dry weight).

BACTERIAL BIOMASS

Bacterial biomass on leaves was estimated by cell counting with epifluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI). One or two leaf disks per each replicate were preserved with 2% formalin and stored until analysis. Samples were sonicated (40 W, 40KHz, Selecta, Spain) for two 2-min cycles to detach bacteria from the leaf, and the cells were dislodged by vortexing for two minutes (IKA® VORTEX, Genius 3, Sigma-Aldrich). Suspensions were properly diluted in a 0.05 M pyrophosphate solution (sodium pyrophosphate decahydrate solution, SIGMA) to avoid aggregates and separate attached cell from the detritus (Velji and Albright 1986). Samples were then incubated for 20 min with DAPI (2 μg $mL^{-1}$) in dark on a shaker to stain the cells. Stained samples were filtered through black polycarbonate filters (0.2 μm, Whatman). Filters were mounted between two drops of immersion oil on grease free slides, covered with cover slips, and bacterial cells counted using a fluorescence microscope (Eclipse E600, Nikon, Japan). Thirty fields were counted for each filter. Bacterial biomass in terms of carbon was estimated as $2.2 \times 10^{-3}$ gC $\mu m^{-3}$ (Bratbak and Dundas 1984) and considering bacteria cell biovolume as 0.1 $\mu m^3$ (Theil-Nielsen and Sondergaard 1998).

FUNGAL BIOMASS

Fungal biomass was estimated quantifying ergosterol concentration in leaf tissues according to Gessner (2005). Ergosterol is the major membrane constituent of fungal cells and is considered one of the best descriptors of fungal metabolically active biomass (Gessner and Schmitt 1996, Charcosset and Chauvet 2001, Abelho 2009).

Sets of five or six leaf disks were lyophilized and weighed to the nearest 0.1 mg, and lipids were extracted with KOH-methanol solution, heated at 80 °C for 30 min. The extracts were purified using solid-phase extraction cartridges (Waters Sep-Pak®, Vac RC, tC18, 500 mg sorbent), and then the ergosterol retained in the cartridges was eluted in isopropanol. Ergosterol was quantified by using high-performance liquid chromatography (HPLC analyser Waters corporation, USA), equipped with a LiChrospher RP18 column (25 cm x 4.6 mm, Merck, Darmstadt, Germany). The sample results were compared with external ergosterol standards at 282 nm absorbance detection. Ergosterol was converted to fungal biomass (in carbon units) by using the factor of 5.5 µg ergosterol $mg^{-1}$ fungal dry mass (Gessner and Chauvet 1993) and considering a 43% carbon content in fungal dry mass (Baldy and Gessner 1997).

FUNGAL SPORULATION

One set of 10 leaf disks were placed into 150 mL Erlenmeyer flasks with 80 ml of filtered stream water (0.2 µm nylon filters, Sarstedt), and incubated for 48 hours at 120 rpm (orbital shaker Infors) and 15ºC. Conidial suspensions were then fixed with formaldehyde (Merck) at a final 2% concentration; and mixed with 35 µL Triton X-100 (Merck) at 15% to disperse conidia. Dispersed conidial suspensions were filtered (47 mm diameter, nitrocelullose filters of 5 µm pore size, Millipore), and retained conidia were stained with 0.1% (w/v) cotton blue in lactic acid (Fluka). Conidia were counted and identified under a light microscope (Leica Biomed) at a magnification of 400 x until at least 300 spores counted per sample.

EXTRACELLULAR ENZYME ACTIVITIES

Five potential extracellular enzymes related to leaf litter processing were measured according to the methods of Artigas et al. (2004) and Romaní et al. (2006a). Three hydrolytic enzymes involved in cellulose and hemicellulose degradation: ß-glucosidase, ß-xylosidase and cellobiohydrolase; one hydrolytic enzyme related to the mineralisation of organic phosphorus: alkaline phosphatase; and one oxidative enzyme, phenol oxidase, involved in lignin degradation. The hydrolytic enzymes were measured by using MUF (methylumbelliferone)-linked artificial substrates, while phenol oxidase was measured with L-DOPA (L-3,4-dihydroxyphenylalanine). The specific MUF linked substrates used for the analysis of hydrolytic enzymes were: 4-Methylumbelliferyl-β-D-glucopyranoside for ß-glucosidase; 4-Methylumbelliferyl-β-D-xylopyranoside for ß-xylosidase; 4-Methylumbelliferyl-β-D-cellobioside for cellobiohydrolase; and 4-Methylumbelliferyl-phosphate for phosphatase (all substrates from SIGMA-ALDRICH). The measurement of extracellular enzyme activity by means

of artificial fluorescent linked substrata is based on the release of the fluorescent molecule (MUF) after the action of the specific enzyme, and the fluorescence at the end of the incubation is directly related to the number of links broken by the enzyme (Hoppe 1993).

All the enzyme assays were carried out under saturating conditions, which varied between intermittent and permanent streams. In the Fuirosos stream saturation concentrations were used following Artigas (2008), while in the permanent streams, saturation curves were calculated previous to the experiment to determine the substrate saturating concentration for each enzyme. In Table 2 a summary of the methodology is showed specifying the saturation concentration used in the different studied streams.

Table 2. Enzyme activities measured in this thesis, showing the artificial substrates and the final saturating concentrations used in the intermittent and permanent studied streams. The EC (Enzyme Commission number) is also indicated for each enzyme. NM= not measured.

| Enzyme | Artificial substrate | Concentration intermittent stream | Concentration permanent streams |
|---|---|---|---|
| β-D-glucosidase (EC 3.2.1.21) | MUF-β-D-glucopyranoside | 0.3 mM | 0.3 mM |
| β-D-xylosidase (EC 3.2.1.37) | MUF-β-D-xylopyranoside | 0.3 mM | 0.4 mM |
| Cellobiohydrolase (EC 3.2.1.91) | MUF-cellobioside | 0.3 mM | 0.3 mM |
| Alkaline phosphatase (EC 3.1.3.1-2) | MUF-phosphate | NM | 1mM |
| Phenol oxidase (EC 1.10.3.2 & 1.14.18.1) | L-dihidroxyphenil-alanine (L-DOPA) | 1.5 mM | 5 mM |

One or two leaf disks from litterbag samples retrieved from the stream were used to analyse potential enzyme activities, which were processed on the same sampling day. All enzyme activity assays were performed using filtered stream water (nylon filters 0.2 µm pore size).

For the hydrolytic enzymes, the specific artificial MUF substrate for each enzyme was added to 15 ml falcon vials where 4 mL of filtered stream water were added to the collected leaf disks. Substrates were added to reach the specific final concentration. Control of water activity (without leaf disks) and water fluorescence (without substrate) were performed, and standards of MUF (0–100 µmol/L) were prepared with filtered stream water. Samples, controls and MUF standards were placed on a shaker at room temperature (20 °C) in darkness for 1 h. Thereafter, glycine buffer (0.05 mmol/L, pH

10.4, 1:2 buffer:sample, volume:volume, 4 mL) was added to each sample, control and MUF standards to stop the reaction. Activity was quantified by fluorescence at 365/455 excitation emission wavelengths (Kontron, SFM 25, Germany) for the Mediterranean stream. In the permanent stream experiments, black multi-well plates (Fisher Scientific) were loaded with 200 µL of each sample and MUF standards, after vortexing, and fluorescence was measured at 350/460 nm excitation/emission wavelengths in a Fluoroskan Ascent FL fluorometer (Thermo Scientific).

For the oxidative enzyme phenol oxidase, the leaf disks were incubated with L-DOPA in acetate buffer (pH 5) solution at the saturation concentration, for 2 hours at room temperature, in darkness and under agitation. Blank for each sample (without L-DOPA) to control for absorbance changes due to the mixing of leaves in acetate buffer and control for the activity in the water were incubated together with the samples. Phenol oxidase activity was estimated based on the absorbance measured at 460 nm (Spectrophotometer Shimadzu UV-2401(PC) CE, Kyoto, Japan) in the sample minus the blank. In the permanent stream experiments, colorless multi-well plates (Fisher Scientific) were loaded with 200 µL of each sample, after vortexing, and absorbance measured at 460 nm in a Spectra Max Plus spectrometer (Molecular Devices). Phenol oxidase activity was estimated by dividing the absorbance by the extinction coefficient 1.66 mM (Sinsabaugh and Linkins 1990).

Extracellular enzyme activities were expressed as µmol DIQC (2,3-dihydroindole-5,6-quinone-2-carboxylate, product of the L-DOPA degradation) or MUF per unit of time and dry weight of leaf.

MICROBIAL ASSEMBLAGES

Bacterial and fungal assemblages were analysed with denaturing gradient gel electrophoresis (DGGE), previous DNA extraction and amplification, following (Duarte et al. 2010). To better integrate the material from each replicate to be used for the community composition analysis, 4 freeze-dried disks were taken per bag, each disk was cut in 4 equally-sized pieces, and each piece used as a replicate subsample for DNA extraction. Sets of four halves of freeze-dried leaf disks were processed with UltraClean ® Soil DNA Isolation kit (MO BIO Labotatories, Inc. Carlsbad, California, USA) or soil DNA extraction kit (MoBio Laboratories, Solana Beach, CA) for DNA extraction of intermittent and permanent streams respectively, and following the manufacturer's instructions. Fungal diversity was assessed with the primer pairs ITS3GC/ITS4 (White et al. 1990), which amplify the ITS2 region of fungal rDNA. Bacterial diversity was assessed with the primer pairs 338GC/518 (Muyzer et al. 1993), which target the V3 of bacterial 16S rDNA.

Polymerase chain reaction (PCR) analysis was performed to amplifying DNA sequences, from both bacteria and fungi. Briefly, 1 µL (1-10 ng $\mu L^{-1}$) of DNA extract was mixed with 0.5 µL of each primer (0.4 µM final concentration), 12.5 µL of GoTaq® Green Master Mix (Promega) and 10.5 µL of water supplied with the GoTaq® Green Master Mix with a final volume of 25 µL, in 0.2 mL PCR tubes (Sarstedt). PCR reactions were carried out in a Doppio thermocycler (VWR International, Radnor, Pennsylvania, USA) as follows: 1) initial denaturation for 2 minutes at 95 ºC; 2) 36 cycles of denaturation for 30 seconds at 95 ºC; annealing for 30 seconds at 55 ºC and extension for 1 minute at 72 ºC, and 3) final elongation for 5 minutes at 72 ºC. The PCR products were run on a 2% agarose gel at 80 V for 45 min to check the presence of the desired band (e.g. Fig. 4).

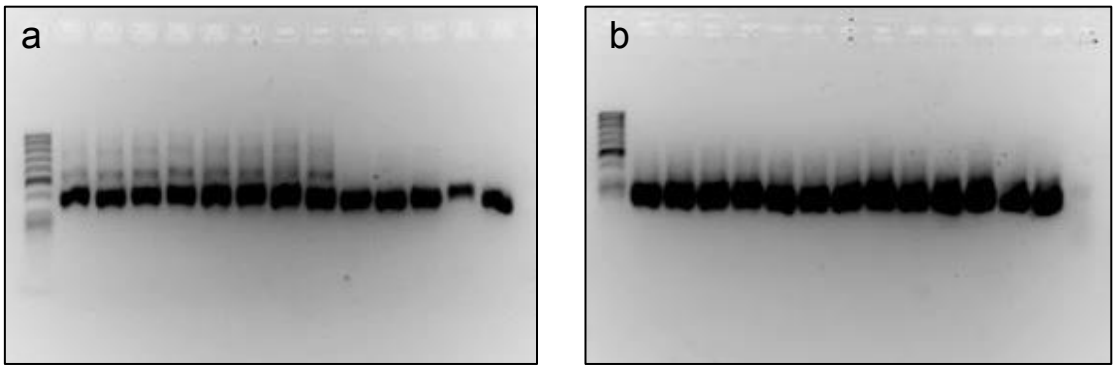



Figure 4. Agarose gel with PCR results for fungal (a) and bacterial (b) DNA from leaf samples collected in the Fuirosos intermittent stream (Chapter II).

DGGE analyses were performed using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). Samples with ca. 750 ng of 380–400 bp for fungal and 200 bp for bacterial amplified DNA were loaded on 8% (w/v) polyacrylamide gels in 1 x Tris-acetate-EDTA (TAE) with a denaturing gradient that varied among intermittent and permanent stream samples. For the samples from intermittent stream denaturing gradients from 30% to 65% for fungi and 45% to 80% for bacteria were used, while in permanent streams the denaturing gradient were from 30 to 70 % or from 45 to 75 % for fungi and bacteria, respectively (100% denaturant corresponds to 40% formamide and 7 M urea). The gels were put to run at 55 V, 56 ºC for 16 h and stained with 1x of GelStar (Lonza) for 10 min. The gel images were captured under UV light in a gel documentation system GenoSmart (VWR, International, Radnor, Pennsylvania, USA). A marker was prepared by mixing equal amounts of DNA of 10 aquatic hyphomycete strains (AA, *Alatospora acuminata* UMB-745.11; AF, *Anguillospora filiformis* UMB-822.11; AT-748, AT-843 and AT-848, *Articulospora tetracladia* UMB-748.11, -843.11 and -848.11, respectively; FP,

*Flagellospora penicillioides* UMB-612.10; LC, *Lunulospora curvula* UMB-578.10; TE, *Tetrachaetum elegans* UMB-717.11; TM, *Tetracladium marchalianum* 440.09 and TC, *Tricladium chaetocladium* UMB-523.10) or DNA of 5 bacterial species (BC, *Bacillus cereus*; PV, *Proteus vulgaris*; SA, *Staphylococcus aureus*; PP, *Pseudomonas putida* and EC, *Escherichia coli*). (e.g. Fig. 5)

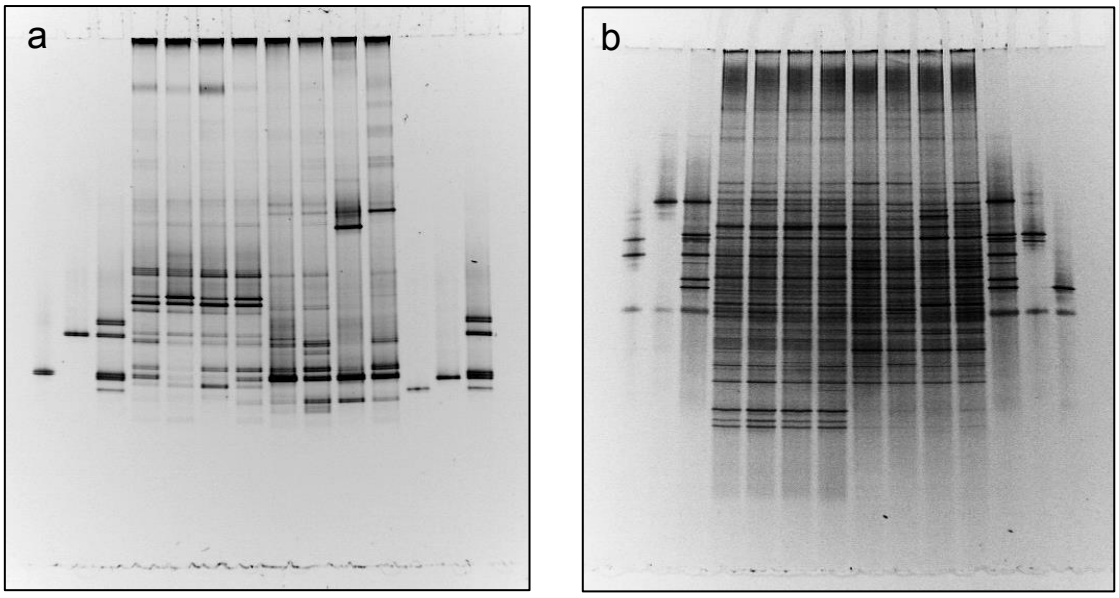



Figure 5. DGGE of fungal (a) and bacterial (b) assemblage from the drought experiment conducted in the Fuirosos intermittent stream (Chapter III).

LITTER QUALITY

Leaves for quality analyses were oven-dried (60°C, 48 h), freeze-dried, ground (with pestle and mortar) and stored in small glass containers until being processed. Two subsamples (2 to 4 mg each) of ground leaves per bag were weight (microbalance Sartorius 2MP to the nearest 0.001 mg) and placed in tin foil crucibles for C/N analysis (CN Elemental Analyser, Perkin Elmer EA2400 Series II), using vanadium pentoxide as oxidation catalyser. A similar weight of ground leaves was analysed for phosphorus content. Samples were digested in basic medium (NaOH) in an autoclave (110 ºC, 90 min; Grasshoff et al. 1983), oxidised to phosphate and quantified using the molybdate method (Murphy and Riley 1962). Fibre, cellulose and lignin content were estimated by gravimetric determination following Gessner (2005b). Briefly, onset with around 200 mg of ground leaf, residual weight of samples was determined following successive removal of tissue constituents using a set of solutions (acid-detergent solution, acetone and sulphuric acid).

C, N, P, fibre, cellulose and lignin content was expressed as percentage of weight and quantity in grams was calculated by multiplying the percentage of content by the remaining weight of leaf.

MACROINVERTEBRATE COMMUNITY

Macroinvertebrate community was analysed in Chapter III. To assess the macroinvertebrate community, the protocol to retrieve litterbags was slightly modified. Litterbags were retrieved from the stream with a tray to catch associated invertebrates. Then, bags were opened and leaves were slightly washed with stream water. Clean leaves were enclosed in plastic bags to be processed in the laboratory. The tray contents were sieved with a 200 µm mesh and invertebrates were collected and preserved in situ in ethanol 70%. All macroinvertebrate specimens were sorted, identified to the lowest taxonomic level possible (genus for most taxa) and sized on a binocular microscope (ZEISS). Biomass was estimated as dry weight, which was calculated from individual lengths using existing equations for macroinvertebrates (Meyer 1989, Smit et al. 1993, Burgherr and Meyer 1997, Benke et al. 1999, Baumgärtner and Rothhaupt 2003). Feeding groups were assigned in base on previous classifications (Merritt and Cummins 1996, Tachet et al. 2000, Monakov 2003) using the dominant or dominants strategies reported for each taxa. Abundance (individuals) and biomass (mg) of invertebrates were expressed per gram of leaf litter.

GENERAL DATA ANALYSIS

The breakdown rates ($k$ day$^{-1}$) were calculated with an exponential decay model (Webster & Benfield 1986).

$$w_t = w_o * e^{-kt}$$

Where $w$ is the dry weight remaining at the time $t$, $w_o$ the estimated initial dry weight and $k$ the breakdown rate coefficient.

Three basic community parameters were calculated for each assemblage (bacteria, fungi and macroinvertebrates): species richness (S); Shannon-Wiener diversity (H) and Pielou's evenness (J) (Pielou 1969). Shannon's diversity and Pielou's eveness indices were calculated as follows:

$$H = -\sum_{i=1}^{s} Pi(\ln Pi)$$

$$J = H'/\ln S$$

Where *Pi* is the relative abundance of conidia or taxon *i* or the relative intensity of OTU *i* or the abundance of macroinvertebrates taxon *i* and *S* is the total number of sporulating taxa, OTUs or macroinvertebrate taxa (Legendre and Legendre 1998).

The specific statistics for each experimental design is described in each chapter.

# Chapter I

# Factors controlling seasonality in leaf litter breakdown for a Mediterranean stream

ABSTRACT

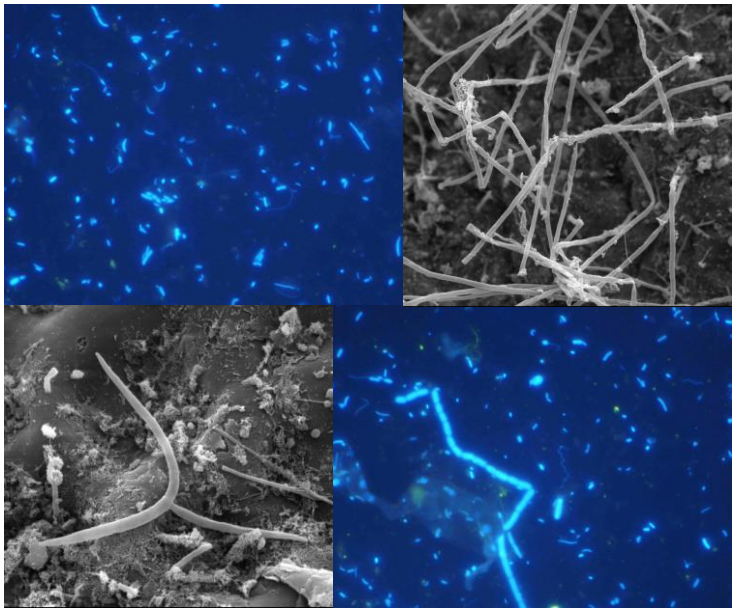
Litter breakdown is a pivotal ecosystem function in headwater streams, where it fuels food webs and controls the carbon flux. Breakdown rates depend on environmental characteristics, and can therefore suffer strong seasonal variation, particularly in intermittent streams, although this topic has been seldom examined. To identify the environmental factors driving seasonality of litter breakdown, we performed five breakdown experiments with poplar leaves during the wet phase (November-August) in a third order intermittent Mediterranean stream. We assessed the contribution of decomposers and detritivores to total breakdown seasonality measuring total (coarse-bag) and microbial (fine-bag) breakdown, and estimating invertebrate-mediated breakdown rates (difference between coarse and fine mesh). Breakdown rates increased from autumn to early summer when expressed in days ($d^{-1}$), and only decreased during the drying phase. However, when expressed in degree-days ($dd^{-1}$), rates peaked in early spring and subsequently decreased. High fine mesh/coarse mesh ratio (0.70) indicated that microbes drove total breakdown. Hierarchical partitioning (HP) analyses in $d^{-1}$ showed that temperature was the most important environmental factor for microbial breakdown, and also affected invertebrate breakdown. Nevertheless, temperature presented strong synergistic effects with other variables. Following removal of the temperature effect, total breakdown was mainly related to current and conductivity, microbial breakdown was related to water quality (conductivity, pH, and oxygen), and invertebrate-mediated breakdown was only related to current. The relation of invertebrate breakdown with current variation might be explained by strong seasonality in total invertebrate and shredder densities, which seemed to be linked to seasonality in discharge. Our results suggest that although on-going climate change can have profound impacts on stream ecosystems, the response of invertebrates and microbes on litter processing and carbon fluxes can be difficult to predict in intermittent Mediterranean streams.

INTRODUCTION

Allochthonous organic matter is a key carbon source in low-order forested streams (Vannote et al. 1980), where food webs are based on detritus (Woodward et al. 2005), and primary production is strongly light-limited (Acuña et al. 2005, Tank et al. 2010). Therefore, particulate organic matter (POM) breakdown is a pivotal ecosystem function (Gessner and Chauvet 2002). Leaves derived from riparian trees are a primary component of allochthonous organic matter; consequently inputs, storage, and breakdown of leaf litter have important consequences for riverine food webs (Gessner

# Chapter II

# Dynamic microbial assemblages and enzyme activities throughout leaf litter decomposition



ABSTRACT

Leaf litter is one of the main constituents of detrital organic matter in streams, and its decomposition is a key ecosystem process mainly driven by microbial and invertebrate activities. The dynamics of microbial decomposition depends on the interaction between different factors, including environmental conditions, taxa present and their ability to colonize the leaves, and the interaction with invertebrates. To understand the seasonal variations in microbial decomposition, we conducted four breakdown experiments with *Populus nigra* leaves (one by season) in a highly seasonal Mediterranean stream, using fine and coarse mesh bag. We measured mass loss, fungal and bacterial biomass, and four extracellular enzyme activities, and characterized bacterial communities with denaturing gradient gel electrophoresis (DGGE). We recognized two stages along microbial leaf decomposition, initial and middle-late stage, which differed in fungal and bacterial assemblages and in their capacity to degrade lignocellulose compounds. During the decomposition process, fungi dominated over bacteria in terms of biomass and in their capacity to decompose lignin. However, contribution of bacteria to total microbial biomass was increased in the initial stage of the process and their assemblages were related with enzyme activities important for polysaccharide degradation. The role of fungi on decomposition was most important in spring, whereas in summer water quality changes seemed to favour bacteria, and litter decomposition was limited by lignin and hemicellulose degradation. Invertebrate presence affected both bacterial and fungal assemblages, stimulating enzyme efficiencies and reducing fungal accumulation in some seasons, possibly related with seasonal invertebrate changes. Our study shows important interactions between fungal and bacterial assemblages and enzyme performance during litter decomposition, and that seasonal variations and invertebrate presence affect litter decomposition beyond breakdown rates.

INTRODUCTION

Detrital organic matter is a key energy source in aquatic ecosystems surrounded by forests (Webster et al. 1990, Wallace et al. 1999, Tank et al. 2010, Marcarelli et al. 2011). In headwater streams, organic matter inputs are dominated by plant litter (Pozo et al. 1997), and are processed by detritivores and decomposers, thus transferring energy and nutrients along food webs (Hieber and Gessner 2002). Plant litter processing in streams is the result of physical abrasion, leaching and biological activities (Gessner et al. 1999, Tank et al. 2010), including fungi and

# Chapter III

# Summer drought affects autumn leaf decomposition in streams

ABSTRACT

Drought is an important disturbance in aquatic ecosystems, especially in intermittent streams. Oncoming changes in land-use and climate will likely increase drought frequency and intensity in many areas, converting permanent streams into temporary, and increasing the duration of drought phases in temporary streams. In Mediterranean region, when streams dry out in summer the leaves that fall into the dry streambed are exposed to solar radiation and high temperatures, which can alter chemical and physical composition of leaves, with impacts on leaf litter decomposition once the water flow returns in autumn. We assessed the consequences of exposing *Populus nigra* leaves to sunlight and high temperature on leaf litter decomposition in autumn, and associated benthic communities (bacteria, fungi and macroinvertebrates). Recently fallen leaves were separated into two sets: control leaves were kept air-dry in the dark, and drought-treated leaves were exposed to simulated summer drought conditions for 79 days. Control and drought-treated leaves were then enclosed in coarse-mesh bags and immersed in a stream (Fuirosos, Spain) for 37 days. Summer drought exposition affected leaf quality by increasing the proportion of nitrogen, total fibre, lignin and cellulose contents in drought-treated leaves. After bags immersion, decomposition process differed between control and drought-treated leaves. Cellulose-degrading enzyme activity was higher in drought-treated than in control leaves, while lignin-degrading enzyme activity was greater in control leaves. Fungal and bacterial assemblages clearly differed between control and drought-treated leaves, showing significantly higher diversity in drought than in control leaves. Moreover, lower variability among replicates in drought-treated leaves was observed in all studied community parameters (biomass and assemblages composition) for bacteria, fungi and macroinvertebrates, suggesting that decomposer and detritivores could be structurally homogenized when leaves were pre-exposed to drought. Despite the drought effect observed on leaf quality, and decomposers and detritivores, breakdown rate of drought-treated leaves did not significantly differ from control leaves. Our study showed a clear effect of drought exposure on leaf litter quality, on the communities colonizing it, and on extracellular enzyme activities, although their joint effect on litter breakdown was weak and statistically non-significant. Nevertheless, this study suggests increased drought could have important effects on the structure and functioning of stream ecosystems, as a consequence of the observed effects at the main basis of the food web in these ecosystems.

# Chapter IV

# Emersion affects leaf litter microbial processing in a pristine temperate stream

ABSTRACT

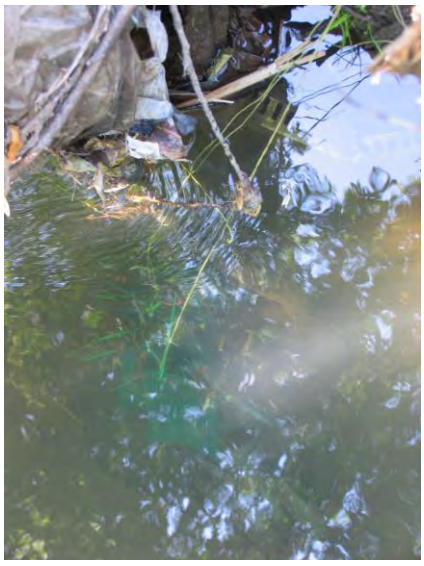
Current global change scenarios forecast an increase in drought frequency and intensity in some temperate regions, which might have severe repercussions on stream ecosystem communities and processes. However, there is still little knowledge about the response to drought of microbial decomposer communities and key ecosystem processes such as litter decomposition. We tested the effect of emersion on microbial decomposers and breakdown of *Populus nigra* leaf litter in a temperate stream. We measured structural (biomass and assemblage composition) and functional (extracellular enzyme activities and sporulation) responses of fungi and bacteria exposed to emersion for different time periods (0 –control–, 7, 14 and 21 days). In general, microbial assemblages and litter breakdown were impacted by emersion, but the response differed between variables. Breakdown rate and activity of β-glucosidase, cellobiohydrolase and phosphatase were progressively delayed with increasing emersion time, while β-xylosidase and phenol oxidase were similarly impacted after the first 7 or 14 days of emersion. Microbial biomass and sporulation showed a threshold of 14 and 7 days of emersion respectively, before differing significantly from the control. Microbial assemblages were affected by the duration of emersion, being fungal sporulation more affected than total fungal assemblages assessed by DGGE analysis. The shifts in fungal assemblages might determine the decrease in microbial capacity to degrade lignin and hemicellulose. Our study shows strong changes on structural and functional aspects of microbial decomposers due to emersion, which might be expected to affect not only the overall decomposition of plant material and the carbon cycle in streams, but also associated invertebrate communities.

INTRODUCTION

In a context of climate change, drier climatic conditions are forecasted in some temperate regions (Beniston et al. 2007, IPCC 2013). Increased air temperatures, reduced precipitations and intensive water usage (e.g. irrigation, drinking water) would affect stream hydrology by increasing the frequency, intensity and duration of droughts (Meyer et al. 1999, Andersen et al. 2006). Although drought is a natural disturbance in streams from semi-arid regions such as the Mediterranean, under current global change trends it will become more frequent in many temperate streams (Acuña et al. 2014). In these streams, biological communities are not evolutionary adapted to drought which threatens its ecological integrity (Schlief and Mutz 2011, Dieter et al. 2013).

# Chapter V

# Eutrophication alters responses of stream-dwelling microbial decomposers to drought



ABSTRACT

A consequence of drought in streams is the emersion of decomposing leaves, which might affect decomposition process and thus organic matter cycling in streams. In this study, we assessed the effects of different emersion time periods (7, 14 and 21 days) on decomposition of poplar leaves and attributes of associated microbes (microbial biomass, extracellular enzyme activities and microbial assemblages) in two streams of NW Portugal differing in the trophic status. The eutrophic stream had lower leaf breakdown rates, fungal biomass and extracellular enzyme activities, but higher bacterial biomass and fungal conidial production than the oligotrophic stream. Microbial richness did not differ between streams, but the structure of fungal and bacterial assemblages was different as suggested by both traditional and molecular techniques. Emersion of decomposing leaves strongly affected all functional measures analysed, but these effects appeared to be different between streams. While leaf decomposition and enzyme activities were more sensitive to emersion in the oligotrophic stream, fungal conidial production and bacterial biomass were more affected in the eutrophic stream, as suggested by emersion time needed to reduce in 50% maximum functions. Microbial assemblage was not affected in terms of species number, but structure was strongly altered after emersion exposure. Although emersion produced similar effects on microbes decomposing leaves in both streams; the differences in functional responses to emersion between streams might have been a consequence of different initial microbial communities, which presented different susceptibilities to the stress imposed by leaves emersion. Our study supports the need of understanding effects of global change in streams suffering from different environmental perturbations such as drought, since responses appear to be influenced by the environmental context.

INTRODUCTION

Human activities are influencing the climate worldwide with impacts on biodiversity and ecosystem processes (Dudgeon et al. 2006, Allan and Castillo 2007). Freshwaters are amongst the most endangered ecosystems mainly due to overexploitation of resources, habitat fragmentation and pollution, which are leading to deterioration and loss of ecosystem services (Dudgeon et al. 2006, Vörösmarty et al. 2010). Climate change poses an additional threat to freshwaters (Allan and Castillo 2007) because of the predicted increase in global surface temperature and shifts in the precipitation regimes (IPCC 2013). Almost all streams and rivers suffer occasional dry periods with flow interruption causing temporary loss of aquatic habitats (Lake 2003, Bruder et al. 2011). A decrease in long term average precipitation and runoff as well as

# General Discussion

Plant litter decomposition is a fundamental process in the carbon cycle, one that affects the structure and dynamics of ecosystems. In low order forested freshwater ecosystems leaf litter is the main source of carbon and energy and its decomposition is determined by both abiotic and biotic mechanisms (Webster and Benfield 1986, Petersen et al. 1989, Gessner et al. 1993, Abelho 2001, Tank et al. 2010). In intermittent Mediterranean streams, flow and environmental conditions change dramatically throughout the year, and often two distinctive phases can be distinguished: a wet phase and a summer dry phase (Gasith and Resh 1999, Lake 2003, Williams 2006, Larned et al. 2010). These particular environmental characteristics affect biological communities and stream functioning (Sabater et al. 2008, von Schiller et al. 2011, Romaní et al. 2012, Vazquez et al. 2013), and potentially modulate leaf litter breakdown across an annual cycle.

Global environmental change, the consequence of increased human population on land uses, water utilization, and global environmental warming, is threatening freshwater ecosystems health and water availability (Palmer et al. 2008, Hamilton et al. 2010). Among the factors relevant for stream functioning, global change is having a strong effect on hydrology, temperature and nutrient availability, as well as on pollution and biodiversity. Scenarios of global warming for the next century, predict reduced flow and enhanced water abstraction in the Mediterranean basin (Beniston et al. 2007, IPCC 2013), as well as increased water scarcity and stronger drought in many temperate streams (Acuña et al. 2014).

The three first chapters of this thesis show that both seasonal variation in the wet phase and exposition of leaves to the summer dry phase affect litter breakdown and microbial process in an intermittent Mediterranean stream. In the two last chapters the likely effect of water scarcity in Portuguese Atlantic streams is evaluated, showing that litter decomposition and microbial assemblages are affected by leaf emersion, and that communities from pristine streams are more sensitive to drought than those from streams previously exposed to anthropogenic eutrophication.

By integrating data from different chapters, in this general discussion we look insight into three aspects: a) the leaf litter decomposition in intermittent Mediterranean streams by connecting the changes in the environmental conditions to the changes in the microbial functioning and assemblages, and the seasonality of leaf litter inputs; b) the effects of drought on leaf litter decomposition, comparing and integrating the effects of summer drought and the effects of emersion; and c) analysing the process for *Populus nigra* L. decomposition since this specific species has been used for all the experiments performed in the thesis.

LEAF LITTER DECOMPOSITION IN INTERMITTENT MEDITERRANEAN STREAMS

Along the wet phase in the intermittent Mediterranean stream (Fuirosos), water characteristics showed strong seasonal variations, mainly driven by air temperature and discharge. After summer drought, flow is recovered and accompanied by an increase in nitrate concentration. Discharge was fluctuating through autumn, winter and spring, and then decreased progressively from spring to summer, and resulted in decreased oxygen concentration and pH, and increased conductivity (Fig. 1). In Chapter I we determined that temperature, flow (current) and water quality parameters (pH, oxygen and conductivity) are the main factors controlling the seasonality of leaf breakdown during the wet period, through a differential seasonal response of microbes and invertebrates.

Biotic influence on litter breakdown seasonality might be related with seasonal shifts in decomposers and detritivores communities, and/or environmental influence on their degradation capacity (Suberkropp 1984, Menéndez et al. 2003, Nikolcheva and Bärlocher 2005, Benstead and Huryn 2011). Our results about microbial decomposition in the Fuirosos stream (Chapter II) suggest seasonal variations in microbial breakdown to result mainly from environmentally-mediated changes in decomposer activity, whereas changes in microbial assemblages were relatively minor. The observed seasonal variation determined few changes in microbial assemblages: bacterial assemblage was quite constant throughout the year and fungal assemblage shifted only in summer, whereas microbial efficiencies to decompose principal leaf compounds varied similarly to leaf breakdown (Fig. 2). In contrast, seasonal variations in invertebrate contribution to decomposition seem to be mainly determined by changes in shredder abundance (Mas-Martí, unpublished, Chapter I), peak densities and peak breakdown rate occurring in spring in the Fuirosos stream.

Fuirosos stream is highly variable among seasons but also among years (Romaní et al. 2006b, Acuña et al. 2007, Sanpera-Calbet 2014), as is typical in the Mediterranean region. Comparing leaf litter breakdown rates from November to December in 2009 (Chapter I) and from November to December in 2011 (Chapter III), this inter-annual variability is very clear. Poplar leaves were decomposed in autumn 2009 at a rate of 0.0074 g DWper day, while in autumn of 2011 it was of 0.049 g DWper day. This remarkable difference is probably related to the strong flood occurring in autumn 2011, which was more than six-fold higher (12955 L $s^{-1}$) than the discharge peak recorded from 2009 to 2010 (Fig. 1). This result suggests that large floods can have an unusual effect on breakdown rate, probably as a consequence of strong physical fragmentation.

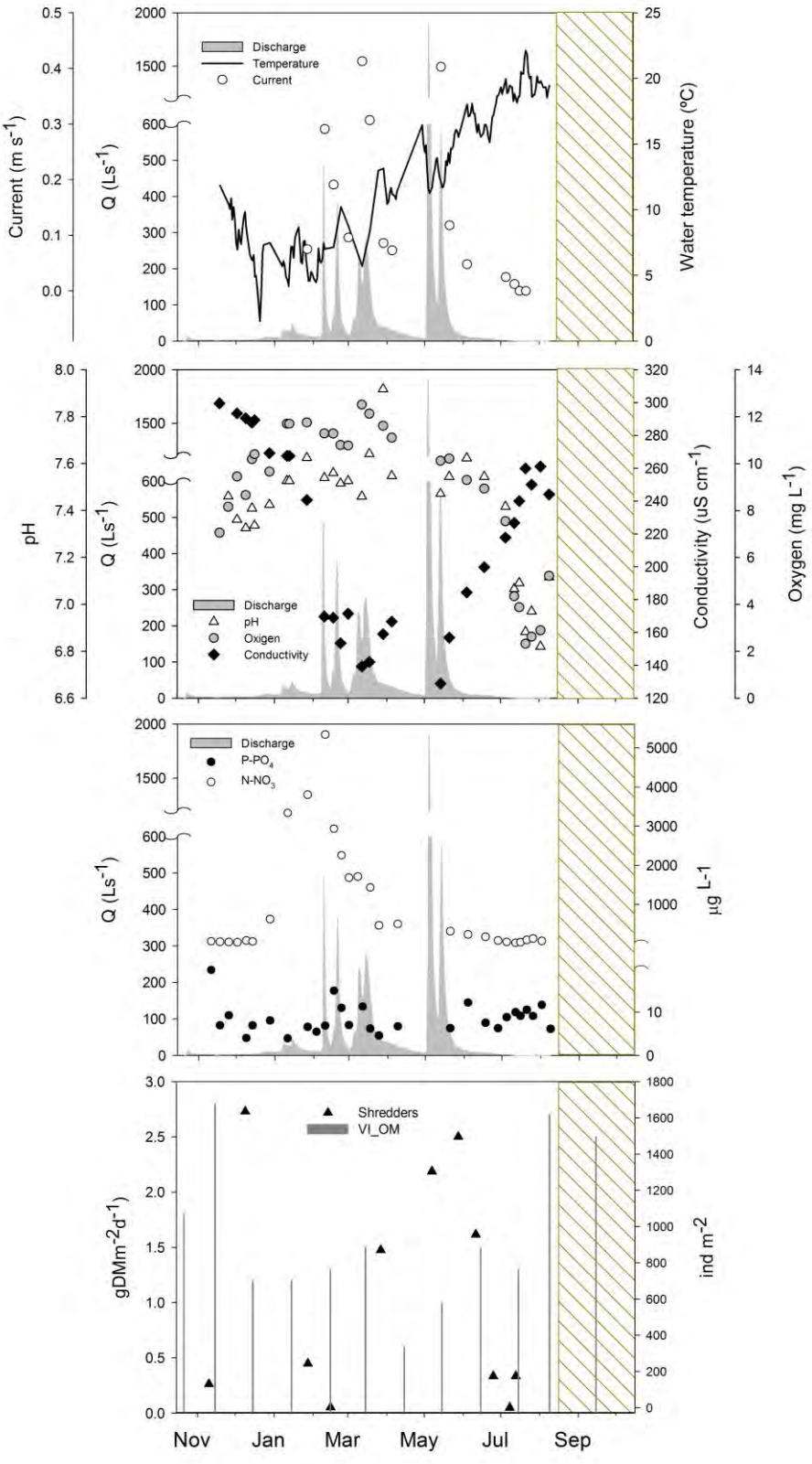



Figure 1. Variation of environmental and biotic variables in the Fuirosos stream throughout the year. Pysical and chemical variables measured in the present thesis, from November 2009 to October 2010 (Chapter I), and biotic variables obtained from previous studies: vertical inputs (VI_OM) of CPOM (Coarse Particulate Organic Matter) (Sanpera-Calbet 2014) and shredder density (Mas-Martí, unpublished data). The striped area indicates the dry phase.

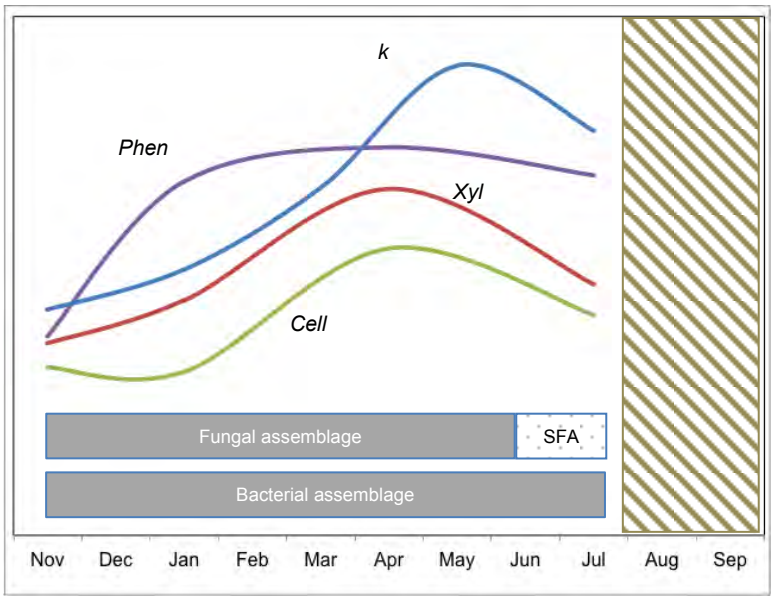



Figure 2. Schematic representation of seasonal changes in poplar litter breakdown rate (*k*), microbial assemblages and efficiency of cellobiohydrolase (Cell), β-xylosidase (Xyl) and phenol oxidase (Phen), in the Fuirosos intermittent stream. SFA: summer fungal assemblage. The striped area indicates the dry phase.

Litter breakdown is a key process to recycle nutrients entering into an ecosystem in form of organic matter (Tank et al. 2010). Taking into account the results of the Chapter I and the pattern of vertical CPOM inputs reported for the studied stream (Sanpera-Calbet 2014, Fig. 1), a mismatch between input and processing might be occurring in Fuirosos stream (Fig. 3). In years with a summer drought period, as the one studied here, vertical inputs follow a bimodal pattern (Sanpera-Calbet 2014) with maxima caused by summer water stress and by autumn leaf abscission; in autumn we measured the lowest breakdown rate and in summer it was reduced. Therefore, the periods of high organic matter inputs do not coincide with the moments of high turnover, as has also been observed in other temperate systems (e.g. Ferreira et al. 2013). This mismatch could locally reduce the total metabolism of carbon and increase the downstream OM exportations, mainly in autumn.

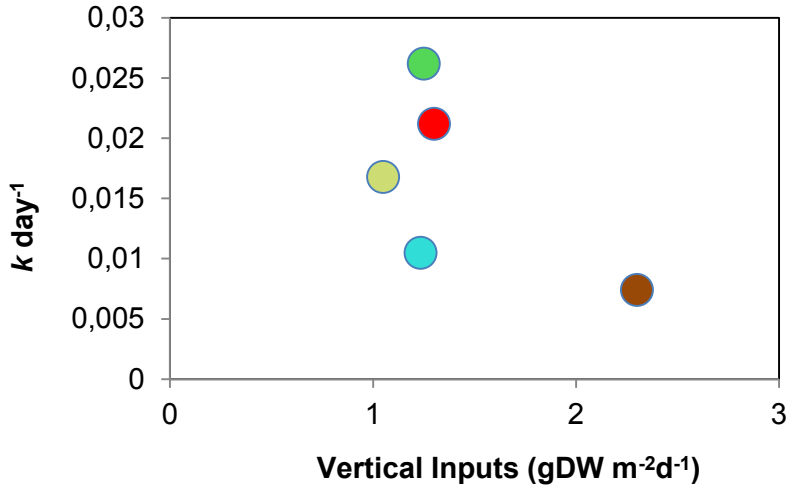



Figure 3. Relationship between the decomposition rate of poplar leaves (Chapter I) and the vertical inputs (VI) of CPOM into Fuirosos stream (from Sanpera-Calbet 2014). Colour legend: autumn in brown, winter in blue, early-spring in yellow, late-spring in green and summer in red.

IMPACT OF DROUGHT ON LEAF LITTER DECOMPOSITION

Drought in streams is a complex process that can have important consequences on organic matter processing (Pinna and Basset 2004, Datry et al. 2011, Corti et al. 2011, Dieter et al. 2011), affecting leaf decomposition through different pathways. We evaluated three possible effects of drought on leaf litter breakdown: 1) effect of pool formation at the initial phase of drought (Chapter I, summer results), 2) effect of exposition of leaves to a simulated intense summer dry phase (Chapter III), and 3) effect of emersion on decomposing leaves, as occur in some summers (Chapter IV). In Table 1 the main effects of drought are summarised.

Overall, this thesis demonstrates that drought produces important shifts in microbial assemblages, affecting microbial growth, enzyme performance and litter breakdown. Decomposition of leaves in summer stagnant conditions is decelerated, likely because of high sensitivity of fungi to the harsh environment (shift in fungal assemblages and biomass reduction), and of low enzyme efficiencies for decomposing lignin and hemicellulose. Emersion of decomposing leaves also reduced litter breakdown rate and all enzyme activities, but fungi and bacteria differed in their sensitivity to emersion. Thus emersion reduced fungal growth and slightly affected fungal assemblage, whereas bacterial assemblages were clearly impacted but biomass accumulation was rapidly recovery after re-immersion. However, sporulating fungal species responded to emersion probably favouring the dominance of amphibious taxa. Finally, although exposition of leaves to intense summer drought seemed not to change litter breakdown velocity once the flow was resumed in autumn, summer drought changed leaf composition increasing cellulose and lignin content, as well as affected bacterial and fungi assemblage and metabolic route used by microbial

community to decompose the leaf (shift from high lignin degradation to high cellulose degradation). Moreover, summer drought effects on leaf quality might homogenise decomposer and detritivore communities, as was indicated by the low inter-replicate variation found for the measured biotic parameters.

Table 1. Summary of drought effects on leaf litter decomposition, microbial processes and invertebrate-mediated breakdown found throughout the experiments performed in this thesis. Glu: β-glucosidase, Cell: Cellobiohidrolase, Xyl: β-xylosidase, Phen: phenol-oxidase. [1]fungal assemblage assessed with DGGE analysis. [2]fungal assemblage assessed with conidia counts. ✔ indicate changes and ✖ no change in the microbial assemblate. = indicates similar values, ↑ increase and ↓ decrease of a particular variable. LDWR: leaf dry weight remaining.

| VARIABLE | BREAKDOWN IN SUMMER POOLS (Chapter I and II) | AUTUMN BREAKDOWN AFTER A DRY PHASE (sunlight & high temperature, Chapter III) | SIMULATED EMERSION IN A PRISTINE ATLANTIC STREAM (Chapter IV) | |
|---|---|---|---|---|
| | | | OVERALL EFFECT | RE-IMMERSION RESPONSE |
| Litter breakdown | ↓ | = | ↓ | = (LDWR) |
| Leaf quality | | ✔ | | |
| Fungal assemblage | | ✔ | ✖[1] ✔[2] | |
| Bacterial assemblage | ✖ | ✔ | ✔ | |
| Fungal Biomass | ↓ | = | ↓ | ↓ |
| Bacterial biomass | ↑ | = | ↓ | = |
| Enzyme production | ↑Xyl, Cbh & Phen | ↑Cbh & ↓Phen | ↓ All | ↓ Glu, Cbh,Phen, ↑ Xyl |
| Enzyme efficiency | ↓Phen & Xyl | | | |
| Sporulation | | | = | ↓ |
| Invertebrate mediated breakdown | ↓ | | | |
| Invertebrate assemblage | | ✖ | | |
| Invertebrate biomass | | ✖ | | |

Interestingly, the responses observed from the different experiments are not equall and for example, while in the Fuirosos decomposition in summer pools bacterial biomass was a relevant fraction of total microbial biomass, bacterial biomass was reduced due to emersion in the Oliveira stream. This might indicate that differential conditions during the drying-drought-rewetting process may either favour or inhibit the development of organisms. Also, differences in the responses could be linked to the different climatic history of these two streams: a Mediterranean stream annually submitted to a summer drought in contrast to an Atlantic stream affected by some flow reduction periods. However, some tendencies can be highlighted by this comparison such as the reduction of the phenol oxidase activity, and thus, of the lignin degrading

capacity when leaves are re-immersed after drought (autumn breakdown after summer drought in Fuirosos and re-immersion after emersion period in Oliveira).

Drought as a perturbation consists in two parts: the disturbance and the biotic responses to the disturbance, which depend on the capacity of the biota to withstand the drought (resistance) or to recover from the drought (resilience) (Lake 2003). Thus, under a disturbance on an ecosystem, microbial composition and the process in which microbes are involved might be affected in different manners (Fig. 4). Microbial composition might be resistant to the disturbance, and not change, and neither alter the ecosystem process; or be sensitive and change, but be resilient and quickly recover to its initial composition without affecting the process, or be sensitive, no resilient, but not affecting the process due to functional redundancy; and finally, be sensitive, not resilient and affecting the process (Allison and Martiny 2008).

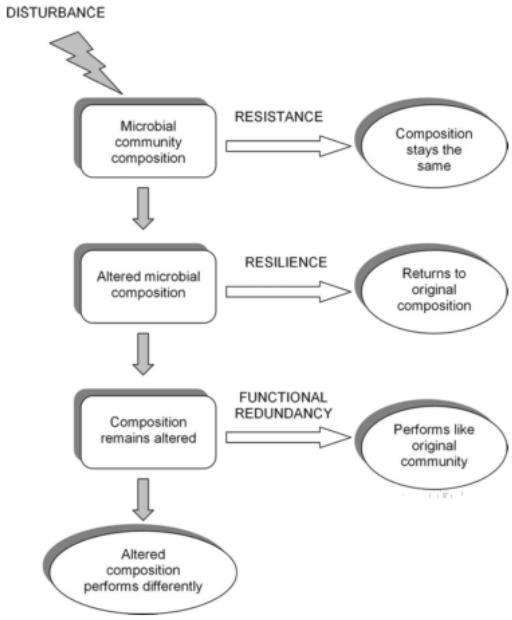



Figure 4. A scheme of how disturbance can change microbial composition and thereby affect ecosystem processes (Allison and Martiny 2008).

Based on the results of this thesis, we found microbial decomposers to be sensitive to drought disturbance, but depending on the specific drought factor, the relevance of microbial changes on leaf litter breakdown varies. Therefore, microbial communities might be in some way resilient (i.e. recovering of some community descriptors after exposition to emersion), show functional redundancy (i.e. microbial assemblage shifts but not breakdown rate affectation, after summer drought exposition of leaves), or be complete affected by drought (i.e. assemblages shifts and breakdown deceleration under pool conditions).

MICROBIAL LITTER DECOMPOSITION OF POPLAR LEAF LITTER

In this thesis, poplar leaves have been used for all experiments and, thus, we expect to be able to infer some general information of its decomposition. Thus, taking into account that leaf species, determining its chemical composition and quality, is a key internal controlling factor of leaf breakdown (Ostrofsky 1997, Leroy and Marks 2006), a question arises: are decomposition and microbial assemblages in decaying poplar similar in permanent Atlantic streams and in Mediterranean intermittent streams?

In Chapter II we observed that for the Mediterranean stream poplar decomposition followed two distinct stages that were consistent through seasons. The initial stage covers the first two weeks of decomposition (to reach around 75% of the leaf mass remaining), presents a characteristic bacterial and fungal assemblage, proportionally higher degradation of polysaccharides and lignin than cellulose, and a higher proportion of bacterial biomass. The middle-late stage (i.e., until 40% of the leaf is remaining) presents also distinctive fungal and bacterial assemblages, higher enzyme production and microbial biomass, increased lignin degradation mainly associated to fungal assemblages, and increased polysaccharide degradation mainly associated to bacterial assemblage.

The comparison between the fungal and bacterial assemblages during poplar decomposition at the Oliveira and the Este showed that stream characteristics are more determinant for microbial assemblages than leaf quality in Atlantic streams (nMDS analysis, Fig. 5). However, changes in the microbial assemblage throughout the decomposition process showed a different pattern between Oliveira and Este streams (Fig. 5a,b). In Oliveira, both bacterial and fungal assemblages progressively changed during the decomposition process from 70% to 20% of litter mass remaining. In contrast, in Este, the bacterial assemblages did not show a clear temporal pattern, and only the initial fungal assemblage (at 70% of leaf remaining) was distinct to that observed in the rest of the process.

Although we cannot make an integrative analysis of the microbial assemblages for the three studied streams (since the DGGE analysis used to assess the microbial assemblages do not allow us to extrapolate species that are not present in the same gel), the comparison between the ordination for the different streams indicates that fungal assemblages followed similar patters during decomposition in both the Oliveira and Fuirosos (Fig. 5c,d). At the Este, the colonisation patterns could be altered by exposition to high nutrient conditions in the stream. Enrichment of freshwater ecosystems affect microbial communities associated with leaf litter decomposition and the velocity in which leaves are processed (e.g. Chung and Suberkropp 2008, Duarte

et al. 2009, Woodward et al. 2012), and our results suggest that also microbial colonisation patterns could be affected, probably due to changes in microbial community present in the stream and/or to an increase in total nutrient availability for microbial processing.

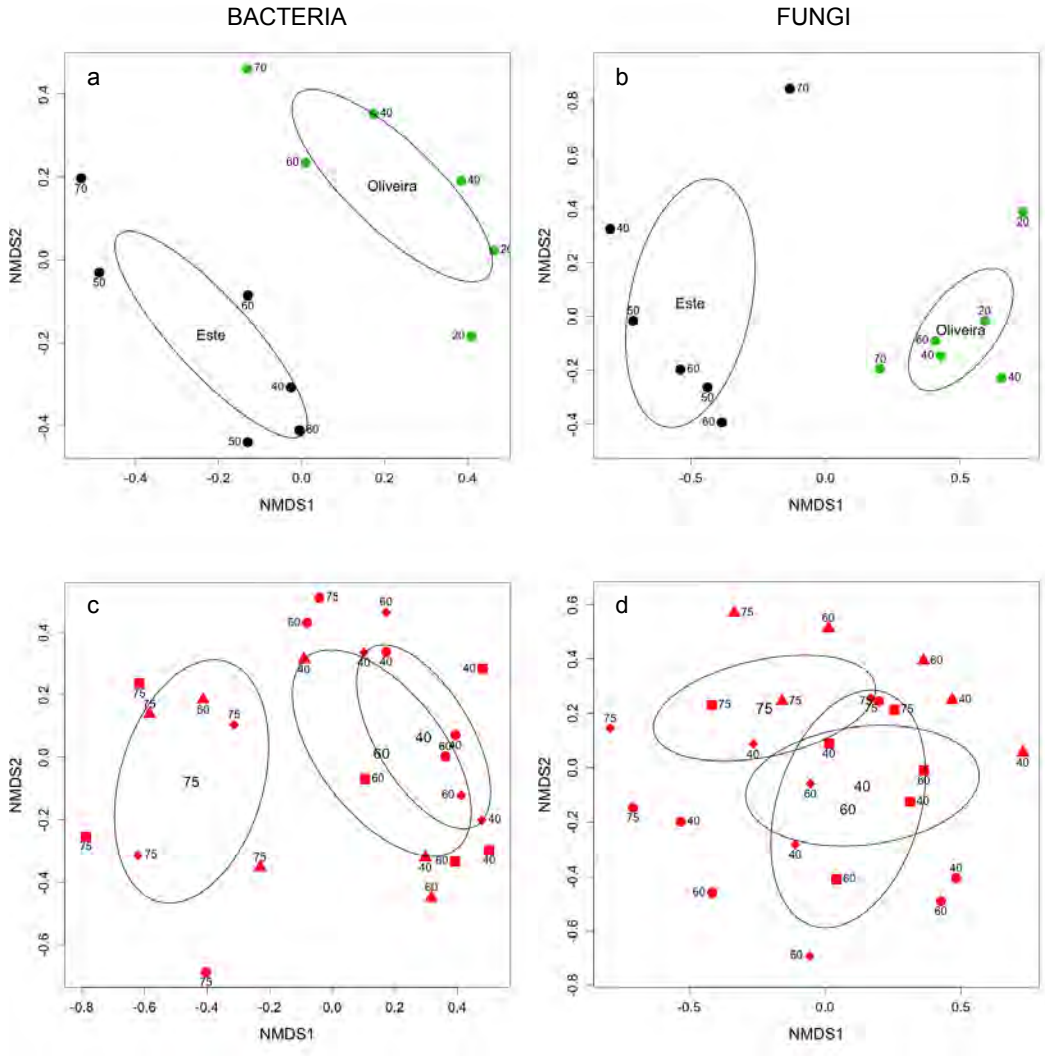



Figure 4. nMDS analysis based on DGGE results for bacterial (left) and fungal (right) assemblages of poplar leaves decomposing in the Atlantic permanent streams Este and Oliveira (a, b), and in the Mediterranean intermittent stream Fuirosos (c, d). The point labels are the leaf mass remaining (%) corresponding to each sample. Shapes in the Fuirosos nMDS correspond to different seasons: autumn in squares, winter in diamond, spring in circles and summer in triangles. The ellipses indicate 95% confidence around their centroids for stream (a,b) and phase of litter processing (75%, 60% and 40%).

Additionally, the integration of the enzyme activities (expressed as proportion of total enzyme production) and the microbial biomass measured through the microbial colonisation, for the studied streams, reinforced the importance of the stream characteristics in the microbial processing of poplar leaves (Fig. 5). Microbial processing in Oliveira seems to be related to higher values of fungal biomass and production of cellobiohydrolase than in the Este and Fuirosos, and this was probably

responsible for the higher breakdown rates in this stream (mean breakdown rates were: Fuirosos: 0.0074 - 0.026 *k* day$^{-1}$, Este: 0.017 *k* day$^{-1}$, Oliveira: 0.047 *k* day$^{-1}$).

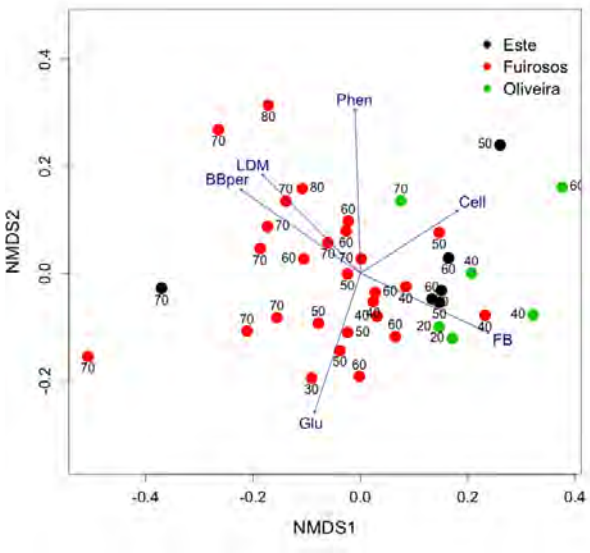



Figure 5. nMDS analysis of proportional enzyme activities and microbial biomass along decomposition process of poplar leaf in three studied streams, one Mediterranean intermittent (Fuirosos) and two permanent Atlantic streams (Este, eutrophic; and Oliveira, oligotrophic).

This figure indicates that the enzyme functioning and microbial biomass will be different depending on the specific site where the poplar leaves are being decomposed, specially separating the Fuirosos from the permanent streams (Fig. 5). However, the tendency of higher lignin degradation and bacterial proportion at initial stages of decomposition observed in the poplar breakdown in the Fuirosos intermittent stream can be still observed when all the data are analysed (Fig. 5). This pattern indicates that initial degradation of lignin and higher development of bacteria occurs, which might be characteristic for decomposition at least for poplar leaves.

## FUTURE PERSPECTIVES

In the recent years, interest of researchers to intermittent streams has been increased likely because of global tendency of water scarcity and the risk of many permanent streams to shift to be intermittent, this being evident in Mediterranean regions (Sabater et al. 2001, Butturini et al. 2003, Pinna et al. 2004, Acuña et al. 2005, Ylla et al. 2010, Romaní et al. 2012). Mediterranean intermittent streams undergo strong environmental variations, through the year and among years, however the difficulties in time and funding often limit the development of long term studies. In this thesis we assessed the influence of environmental factors on leaf litter breakdown through a year and observed evidence of important seasonal variation as well as

showing inter-annual variation. However, the length of this study did not allow us to test the inter-annual variation which we believe that will be relevant to be investigated.

One of the most determinant periods of the hydrological cycle in this type of streams is the summer drought, which is characterised by an initial flow reduction, habitat disconnection and formation of pools, followed by the dry phase and further flow recover (Lake 2003). Studies about the consequences of this period on biological communities and processes are numerous, however there are many factors interacting and conditions during this period are very fluctuating, this making difficult to understand the overall effect of drought on the system. In this thesis we tried to cover some of the possible effects of the stream drought on leaf breakdown, nevertheless many questions remain still unsolved. For example, in the pools there are many factors that shift at the same time and that have differential known effect on leaf decomposition: increased temperature in pools might accelerate microbial decomposition (Dang et al. 2009, Ferreira and Chauvet 2011b), while oxygen depletion and reduced pH might decelerate them (Medeiros et al. 2009, Simon et al. 2009). At the same time, in pools it is possible to find high concentrations of humic acids due to leaching of accumulated leaves in the pools, which also might affect the decomposition of leaves (Canhoto et al. 2013). Thus, all these factors are interacting at the same moment but their relevance may depend on the pool characteristics (e.g. size, time of formation, deep). Some laboratory experiments controlling these factors or extensive sampling studies could be useful to test this combined effects and understanding which processes are driving and which mechanisms are occurring during leaf processing in the pools.

Finally, regarding to the role of fungal and bacterial communities on the microbial processes involved in leaf decomposition further questions arises from the results of this thesis. It is hypothesised that fungal community (specially aquatic hyphomycetes) is functional redundant in terms of the enzyme capacity to degrade leaves (e.g. Bärlocher 2009, Gessner et al. 2010), however through the experiments performed in this thesis, a relation among the fungal assemblages and enzyme activities was constantly observed. Relationship between structure and function is useful to understand the real effect of the disturbance on ecosystem (Allison and Martiny 2008, Comte et al. 2013). Thus, it will be interesting to investigate in detail the functional traits of the microbial decomposer communities (e.g. enzyme activity capacities, growth requirement, survival strategies), and for this objective molecular tools will be the best option, considering field and laboratory experiment, and different possible disturbances.

# General Conclusions

**CHAPTER I. Factors controlling seasonality in leaf litter breakdown for a Mediterranean stream**

1. Litter breakdown of poplar leaves undergoes important seasonal variations in a Mediterranean intermittent stream. Total litter breakdown of poplar leaves varied through the year increasing from autumn to late spring-early summer and decreasing during the drying phase.

2. Breakdown rates ranged throughout the studied year from 0.0074 to 0.0262 $d^{-1}$ for total breakdown (coarse mesh), from 0.0054 to 0.0178 $d^{-1}$ for microbial-mediated breakdown (fine mesh), and from 0.0020 to 0.0100 $d^{-1}$ for invertebrate-mediated breakdown (coarse minus fine mesh).

3. Temperature, current velocity and water quality (pH, oxygen concentration and conductivity) were controlling the observed seasonality in total breakdown, however environmental factors differentially affected microbial-mediated breakdown and invertebrate mediated breakdown.

4. Microbial-mediated breakdown was mainly driven by temperature, conductivity, and flow velocity whereas invertebrate breakdown was primarily associated with current velocity. The sensitivity of invertebrate breakdown to current variation might be explained by annual seasonality in invertebrate and shredder densities, while microbial-mediated sensitivity to temperature and water quality might be related to microbial activity response to annual environmental variation (mainly linked to summer drought events).

**CHAPTER II. Dynamic microbial assemblages and enzyme activities throughout leaf litter decomposition in a Mediterranean stream**

5. Microbes processed poplar leaves in two distinct stages through decomposition, an initial stage and middle-late stage, which were consistent through seasons.

6. The early stage lasted until 75% of leaf weight was remaining. It was characterized by a high contribution of bacteria to microbial biomass and distinctive bacterial and fungal assemblages. Enzyme activities were low but with higher degradation of polysaccharides and lignin than cellulose.

7. The middle-late stage covered from 60% to 40% of leaf mass remaining. In this stage microbial community grew and fungi dominated over bacteria in terms of accumulated biomass. Distinctive assemblages were also formed both for fungi and bacteria and enzyme production increased. In this stage, fungal assemblage was related to higher phenol oxidase activity while bacterial assemblage was related to an increase in simple polysaccharides and cellulose degradation.

8. β-xylosidase and phenol oxidase showed the highest enzyme efficiency to decompose leaves suggesting that decomposition of hemicellulose and lignin might be limiting steps for degradation of poplar leaves.

9. Temporal dynamics of microbial processes were modulated by seasonal environmental variations. The role of fungi on decomposition was most important in spring, whereas in summer water quality changes seemed to favour bacteria, and litter decomposition was limited by lignin and hemicellulose degradation.

10. Invertebrate presence affected both bacterial and fungal assemblages, stimulating enzyme efficiencies and reducing fungal accumulation in some seasons, possibly related with seasonal invertebrate changes.

**CHAPTER III. Summer drought affects autumn leaf decomposition in streams**

11. Exposition of poplar leaves to summer drought conditions (high temperature and solar radiation) affected leaf quality by increasing the proportion of nitrogen, total fibre, lignin and cellulose content, and reduce litter mass. The changes observed after drought exposition probably are due to the combined effect of leaching, photo-degradation and terrestrial microbial processing.

12. The changes in litter quality produced by exposition to drought affected microbial functioning and the composition of both bacterial and fungal assemblages when the leaves were immersed in the stream.

13. Summer drought exposition of leaves increased cellulose-degrading enzyme activity and reduce the lignin-degrading enzyme activity. However the changes observed did not determine changes in breakdown velocity.

14. Lower variability among replicates in drought-treated leaves was observed for all the studied community parameters (biomass and species composition), suggesting that decomposer communities were structurally homogenized when leaves were pre-exposed to drought.

**CHAPTER IV. Emersion affects leaf litter microbial processing in a pristine temperate stream**

15. Emersion of leaf litter in temperate streams reduces breakdown rate and affects assemblages, extracellular enzyme production and growth of microbial decomposers.

16. Microbial functioning was more sensitive to emersion than microbial biomass growth. Microbial activities were progressively delayed just after emersion and fungal sporulation peak decreased when emersion lasted more than 7 days, whereas bacterial and fungal biomass were affected only after 14 days of emersion.

17. Microbial assemblages were changed by the duration of emersion, being sporulating fungal assemblages more affected than total fungal assemblages. The impact of emersion regime on sporulating species was mainly reflected in changes in the contribution of some fungal species, favouring amphibious fungal species. The shifts in fungal assemblages were related to a decrease in microbial capacity to degrade lignin and hemicellulose under emersion disturbance.

18. Resilience capacity of the different microbial components (measured as the response of the microbial variables after 7 and 14 days of re-immersion) slightly differed from the sensitivity observed. Fungal biomass, fungal sporulation rate and the enzymes involved in lignocellulose compounds remained affected after re-immersion, whereas the enzyme for phosphorus uptake and bacterial biomass were faster recovered once the leaves were re-immersed.

**CHAPTER V. Eutrophication alters responses of stream-dwelling microbial decomposers to drought**

19. Leaf decomposition and associated microbial decomposer differed between the oligotrophic and eutrophic studied streams, as well as their response to emersion exposure of decomposing leaves.

20. The eutrophic stream had lower leaf breakdown rates, fungal biomass and extracellular enzyme activities, but higher bacterial biomass and fungal conidial production. Microbial richness did not differ between streams, but the structure of fungal and bacterial assemblages was different.

21. Leaf decomposition and enzyme activities were more sensitive to emersion in the oligotrophic stream, while fungal conidial production and bacterial biomass were more affected in the eutrophic stream, as suggested by emersion time needed to reduce in 50% maximum values.

22. Microbial assemblage was not affected in terms of species number, but structure was strongly altered after emersion exposure. Although emersion produced similar effects on microbes decomposing leaves in both streams; the differences in functional responses to emersion between streams might have been a consequence of different initial microbial communities, which presented different susceptibilities to the stress imposed by leaves emersion.

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