Universidade do Minho
Escola de Ciências

José Miguel Castro Trabulo

Effects of eutrophication on stream-dwelling decomposers of plant-litter

Master thesis

Master in Ecology

Work made under the orientation of

Prof. Dr. Fernanda Maria Fraga Mimoso Gouveia Cássio

Prof. Dr. Maria Cláudia Gonçalves Cunha Pascoal

Dr. Sofia Alexandra Ferreira Duarte

October 2013

DECLARAÇÃO

Nome: José Miguel Castro Trabulo

Endereço eletrónico: zemiguel.eco@gmail.com

Número do Bilhete de Identidade: 13736725

Título tese: Effects of eutrophication on stream-dwelling decomposers of plant-litter

Orientador: Fernanda Cássio, Cláudia Pascoal e Sofia Duarte

Ano de conclusão: 2013

Designação do Mestrado: Ecologia

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Universidade do Minho, _____/_____/_____

Assinatura: _____

(José Miguel Castro Trabulo)

Acknowledgments

I would like address a few words to the ones who direct or indirectly helped me during this time and contribute to this work:
To Prof. Cláudia Pascoal and Prof. Fernanda Cássio for all knowledge transmitted and all the support during not only in my Masters' second year but also since the beginning of 2011 when I joined the group.

To Sofia, it was a pleasure working with someone I consider a role model and surely the most hard-working, dedicated and committed person I have ever met. Since the first appearance in the lab you've helped me and your guidance was, without doubt, remarkable! Our group is very lucky to have you! To you a huge thanks.

To Cláudia, Sara, Bruno for being there when I needed, but specially to my great friend Francisco Carvalho who has been a huge support during this journey, for all the support in the bad times, but specially for the awesome times we have spent since our first year in college your friendship has been gratifying! They say that true friends appear when you enter in college and I can confirm it! Thank you and I wish our journey is just getting started.

To Eva, for a huge number of reasons built during the past year, for the companionship and for all the help in the lab! Great moments.

To Maria João, Isabel, Daniela, Ana, Arunava, Diana, Carla, Barbara and more recently Ilisa and Mafalda for all the good moments in the lab and for all the help and companionship!

To my Mother and Father because so far you didn't let me down, for not letting me give up from persecute my goals and for having done more than they could, sometimes, to graduate me! Thank you

To my sister who is surely my best friend and a huge support in all my decisions! You always have the right advice to the right time!

To Barbara, for everything!

This study was funded by FEDER-COMPETE (FCOMP-01-0124-FEDER-013954) and FCT (PEst-C/BIA/UI4050/2011, PTDC/AAC-AMB/113746/2009 and FCT PTDC/AAC-AMB/117068/2010)

Effects of eutrophication on stream-dwelling decomposers of plant-litter

Abstract

Plant-litter decomposition is the major source of nutrients and energy in low-order forested streams. Microorganisms, in particular aquatic fungi, play an important role in this process by mineralizing plant-litter and transforming it into a more palatable food source for macroinvertebrates. However, anthropogenic stressors, in particularly eutrophication, can strongly affect aquatic biota with consequences to plant-litter decomposition in freshwater ecosystems.

In this study, we assessed the effects of eutrophication on leaf-litter decomposition and associated biota by analyzing fungal diversity, reproduction and biomass, benthic macroinvertebrate diversity and biomass and decomposition rates of oak leaves in five streams in Northwest Portugal, differing in their trophic status: Agra, oligotrophic; Oliveira and Andorinhas, moderately eutrophic; Selho, highly eutrophic and Couros, hypertrophic streams. In addition, since fungal sporulation is often severally reduced in hypertrophic streams, we employed both traditional (fungal spores identification) and molecular techniques (denaturing gradient gel electrophoresis - DGGE) to assess fungal diversity more accurately.

Eutrophication affected fungal (biomass and sporulation) as well as macroinvertebrate biomass in the 5 streams. Higher leaf decomposition rates and fungal biomasses were found in the moderately eutrophic streams (Oliveira and Andorinhas) than in highly eutrophic streams (Selho and Couros). Higher fungal reproduction was also found in Selho, Andorinhas and Oliveira streams, than in Agra and Couros streams. On the other hand, higher biomasses of invertebrates, particularly Oligochaeta and Chironomidae, were found in the most eutrophic streams. Fungal diversity, assessed as spore identification and counts, was severally reduced in Couros than in the other streams, but DGGE fingerprints of fungal communities revealed in high diversity in all streams. Shredder taxa were dominant in decomposing oak leaves in the less eutrophic streams (Agra, Oliveira and Andorinhas), but these invertebrates were almost or completely absent in the most eutrophic streams (Selho and Couros). The community structure of both fungi (from fungal spores or DGGE fingerprints) and macroinvertebrates discriminated streams according to the level of eutrophication.

Overall results suggested that the combination of structural measures of fungal and invertebrate communities with functional measures, such as leaf-litter decomposition, may help to better assess the impacts of eutrophication in freshwater ecosystems.

Efeitos da eutrofização nas comunidades decompositoras da folhada em rios

Resumo

A decomposição da folhada constitui a principal fonte de nutrientes e energia em ribeiros florestados para os organismos aquáticos. Os microrganismos, em particular os fungos aquáticos, desempenham um papel importante neste processo mineralizando detritos vegetais e aumentando a sua palatabilidade para os macroinvertebrados detritívoros. No entanto, os agentes de stresse antropogénico, em particular a eutrofização, podem afetar os organismos aquáticos e a decomposição da folhada nos ecossistemas de água doce.

No presente estudo, avaliou-se o efeito da eutrofização na decomposição da folhada e nos organismos envolvidos no processo, analisando a diversidade, a reprodução e a biomassa dos fungos; a diversidade e biomassa dos macroinvertebrados, e a taxa de decomposição da folhada de carvalho em 5 rios no Noroeste de Portugal que diferiam no seu estado trófico: Agra, oligotrófico; Oliveira e Andorinhas, moderadamente eutróficos; Selho e Couros, hipertróficos. Uma vez que em rios poluídos a reprodução dos fungos associados à folhada em decomposição é fortemente inibida em rios hipertróficos, foram usados métodos tradicionais (identificação de esporos) e moleculares (electroforese em gel com gradiente desnaturante - DGGE) para avaliar de forma mais eficaz a diversidade dos fungos nos rios.

A eutrofização afetou a biomassa e a esporulação dos fungos a biomassa dos macroinvertebrados nos 5 rios. As taxas de decomposição da folhada e a biomassa dos fungos foram mais elevadas nos rios moderadamente eutróficos (Oliveira e Andorinhas) do que nos rios hipertróficos (Selho e Couros). As taxas de reprodução dos fungos foram mais elevadas nos rios Oliveira, Andorinhas e Selho do que nos rios Agra e Couros. No entanto, a biomassa de invertebrados foi superior nos rios mais eutrofizados devido às biomassas elevadas de Oligochaeta e Chironomidae. A diversidade de fungos, avaliada com base na identificação dos esporos libertados da folhada em decomposição foi severamente reduzida no rio Couros, comparativamente com os restantes locais. No entanto, a diversidade dos fungos avaliada com base no número de unidades taxonómicas operacionais- OTUs, obtidas a partir do gel de DGGE, foi elevada nos 5 rios. Os invertebrados trituradores eram dominantes na folhada em decomposição nos rios Agra, Oliveira e Andorinhas, enquanto nos rios mais eutrofizados, como o Selho e o Couros, estes estavam ausentes ou em número muito reduzido. As comunidades de fungos (avaliadas a partir da identificação dos esporos ou do perfil do DGGE) e de macroinvertebrados foram capazes de discriminar rios de acordo com o grau de eutrofização.

Os nossos resultados sugerem que a combinação de medidas estruturais, das comunidades de fungos e de macroinvertebrados bentónicos, com medidas funcionais, como a decomposição da folhada, poderá melhorar a nossa capacidade de avaliar os impactos da eutrofização nos ecossistemas de água doce.

Table of contents

List of Figures

List of Tables

1. Introduction

1.1. Plant-litter decomposition in freshwater ecosystems

Two important determinants of ecosystem biomass and trophic structures in freshwaters are respiration and primary production, which are crucial drivers of nutrient cycling and ecosystem processes (Mulholland *et al.,* 2001). Respiration provides an indication of total consumption of organic matter provided by autochthonous and allochthonous sources (Allan and Castillo, 2007). Autochthonous organic matter is supplied by photosynthetic production within the stream, while allochthonous organic matter has a terrestrial origin, mainly from the surrounding forest such as leaves, twigs and other parts of the riparian vegetation (Allan and Castillo, 2007).

In small headwater streams, allochthonous organic matter supply is representative of primary production, which is limited by the absence of light and low temperatures due to the shadow from the canopies provided by the surrounding riparian vegetation (Allan and Castillo, 2007). This allochthonous organic matter, in particular the leaves that fall in large quantities in autumn in temperate climates, can be trapped in the stream or transported downstream (Elosegi, 2005), constituting the main source of nutrients and energy to detritus aquatic food webs (Abelho, 2001; Benfield, 1996; Cummins, 1974; Mulholland *et al.,* 2001). The aquatic biota – in particular macroinvertebrate detritivores and microorganisms - constituting these detritus food webs are the major drivers of leaf litter decomposition.

Three distinct phases, which can occur sequentially or simultaneously, are generally recognized during leaf litter decomposition in streams: 1) leaching, 2) conditioning and 3) fragmentation (Allan and Castillo, 2007). The leaching phase corresponds to the rapid loss of litter soluble compounds, such as phenolics, carbohydrates and amino acids in a period of 24 hours to 7 days (Casas and Gessner, 1999; Petersen and Cummins, 1974; Webster and Benfield, 1986). The conditioning or microbial colonization reflects the growth and partial degradation of litter by microorganisms, mainly fungi and bacteria, which increases leaf litter palatability as food source for invertebrate detritivores, through the accumulation of microbial biomass with high nutrient value (Cummins, 1974; Suberkropp, 1998). Mounting evidence suggests that conditioning is very important since invertebrate shredders show preference to feed

on conditioned leaves (Chergui and Pattee, 1991; Graça, 2001; Graça *et al.,* 1993; Kiran, 1996). Invertebrate detritivores seem not to have the enzymatic capability to break the structural compounds of leaves (Bärlocher and Porter, 1986) with some exceptions, e.g., the detritivores that possess endosymbionts in the gut which may be involved in the digestion of cellulose (Klug and Kotarski, 1980; Zimmer and Topp, 1998).

There is a strong relationship between shredder abundance and fungal biomass on leaves (Barlocher and Kendrick, 1975; Graça, 1993) and two explanations for that are: 1) invertebrates benefit from fungal enzymatic activity on leaves and 2) invertebrates can also feed on fungi. The time of microbial colonization of litter varies, but it has been referred to occur within one or two weeks, depending upon the degree of pre-conditioning of the substrate in the terrestrial environment and upon the temperature regime (Cummins, 1974).

The last phase - the fragmentation - occurs as a result of both mechanical and biotic fragmentation. Mechanical fragmentation results from the abrasion and shear stress exerted by the flowing water (Gessner *et al.,* 1999), while biotic fragmentation is generally associated with the breakdown of leaves – the coarse particulate organic matter (CPOM) – into smaller pieces – the fine particulate organic matter (FPOM) through the feeding and digestive activity of invertebrate shredders (Cuffney *et al.,* 1990; Cummins, 1974). This FPOM will then constitute an important food source for the invertebrate collectors.

The flow of organic matter during leaf breakdown has a large number of pathways that reflect a number of transformations that can be chemical, biological, and mechanical (Gessner *et al.,* 1999; Petersen and Cummins, 1974; Webster and Benfield, 1986). Microbial activity and leaf decomposition in streams is regulated by several intrinsic and extrinsic factors (Abelho, 2001). An example of an intrinsic factor is litter quality (Royer *et al.,* 2001), with leaves of lower quality (e.g. with more recalcitrant compounds such as lignin) decomposing more slowly than leaves with higher quality. Among the extrinsic factors, temperature (Chauvet and Suberkrop, 1998), concentration of dissolved nutrients (Grattan and Suberkrop, 2001; Shridar and Bärlocher, 2000; Suberkrop and Chauvet, 1995) and pH (Dangles and Chauvet, 2003; Pascoal and Cássio, 2004) can strongly affect litter decomposition. These factors can depress or accelerate the transformation of allochthonous organic matter falling into streams which is transformed into several products, including microbial and invertebrate biomass, FPOM, dissolved organic matter (DOM), inorganic nutrients and carbon dioxide (Gessner *et al.,* 1999).

1.2. Aquatic biota involved in plant-litter decomposition

1.2.1. Macroinvertebrates

According to Cummins (1973), macroinvertebrates can be divided in four functional feeding groups: 1) shredders, 2) collectors, 3) scrapers and 4) predators. Shredders feed on CPOM namely decomposing vascular tissues (Cummins, 1973). This preference may be the result of changes in leaf matrix by the microbial community or by the presence of fungal hyphae of higher nutritive value than the leaves themselves. Invertebrate feeding on leaves leads to the incorporation of plant material into secondary production and to the fragmentation of leaves (Graça, 2001). Collectors feed on FPOM, either by filtering the detritus from the water column - collectors-filterers - or gathering detritus from the substrates - collectors-gatherers. On the other hand, scrapers feed on the biofilm covering submerged structures, such as stones and plant stalks. The last group of macroinvertebrates, the predators, feed on other animals' (Graça, 2001).

Invertebrate shredders are those that most contribute to plant-litter decomposition in streams by transforming CPOM into FPOM, as described above and in the previous subsection. In addition, shredders produce abundant fecal pellets resulting from the incorporation of some nutrients in secondary production (Graça, 2001), that can also constitute an important source of FPOM. The FPOM production can play a very important role in lower reaches of streams for invertebrate collectors (Vannote *et al.,* 1980). In general, shredders emerge earlier in the season than collectors, whose growth is enhanced by the production of FPOM (Dieterich *et al.,* 1997).

Microbial conditioning of leaves appears to be an important process for invertebrate shredders (Golladay *et al.,* 1983; Graça, 2001). Shredders feed preferentially on conditioned leaves and its abundance is positively correlated with fungal biomass on leaves (Chergui and Pattee, 1991; Graça, 2001; Kiran, 1996, Robinson *et al.,* 1998). Furthermore, shredders appear to have preference for leaves conditioned by specific fungal species (Graça *et al.,* 1993; Lecerf *et al.,* 2005).

1.2.2. Aquatic fungi

Aquatic fungi, particularly aquatic hyphomycetes are a phylogenetically heterogeneous group of fungi and are the main microbial decomposers of leaf litter in streams (Bärlocher, 2005; Suberkropp, 1998). Aquatic hyphomycetes are reported to affect both autotrophs and heterotrophs (microorganisms and invertebrates) (Bärlocher, 2005; Suberkropp, 1998). By actively growing on plant-litter, aquatic hyphomycetes release nutrients and dissolved organic matter (DOM) that will be available to other heterotrophic microorganisms, such as bacteria. However, competition between fungi and bacteria by plant-litter resources might also occur (e.g. Mille-Lindblom and Tranvik, 2003). Moreover, by increasing fungal biomass, while growing on leaf-litter, and enzymatically transforming recalcitrant plant compounds (e.g. cellulose, hemicelluloses) into more digestible compounds, aquatic hyphomycetes improve leaf palatability to shredders. In addition, the FPOM and DOM released from leaf-litter due to the enzymatic activity of aquatic hyphomycetes will also constitute an important food source to invertebrate collectors (Bärlocher, 2005).

According to Bärlocher (2005) the dominant role played by aquatic hyphomycetes is mainly due to their notable adaptations to aquatic life such as the production of large amounts of sigmoid and tetraradiate conidia, that facilitate adhesion to plant substrates in turbulent waters. These tetraradiate and sigmoid conidia were discovered by Ingold (1942) in foam naturally formed in many running waters, and he showed that the preferred substrates of fungi releasing these conidia were the deciduous leaves that fall into streams (Bärlocher, 2007). Other characteristics of aquatic hyphomycetes are their ability of being active at very low temperatures and of producting lignocellulose-degrading enzymes that break the structural compounds of plant material enhancing leaf palatability.

1.3. Assessing the diversity of aquatic fungi on plant-litter decomposing in streams: traditional versus molecular techniques

The traditional method used for estimating fungal diversity on decomposing plant-litter is the examination of sporulating structures (conidia) either directly on leaf surfaces or released from decomposing leaves, after sporulation induction (Bärlocher and Kendrick, 1974; Bärlocher,

2005; Das *et al.,* 2008; Suberkropp and Klug, 1976). However, the identification of fungal species based on their reproductive ability can miss fungal taxa that are not sporulating (Nikolcheva *et al.,* 2003, 2005).

In the mid of 90's, a fingerprinting technique - the denaturing gradient gel electrophoresis (DGGE) - of PCR-amplified ribosomal DNA fragments has been introduced into microbial ecology (Muyzer *et al.,* 1993) and about 10 years ago, Nikolcheva and collaborators (2003) applied for the first time this technique to study fungal communities on plant detritus decomposing in streams. In DGGE, DNA fragments with the same length but with different nucleotide compositions, can be separated in a polyacrilamide gel that contains a mixture of an increased gradient of DNA denaturants - urea and formamide (Myers *et al.,* 1987). By using DGGE, 50% of the sequence variants can be detected in DNA fragments up to 500 bp (Muyzer and Smalla, 1998; Myers *et al.,* 1985).

In DGGE, during PCR, a GC-rich sequence (GC clamp) is attached to one side of the DNA fragment that will allow only its partial denaturation. This will decrease the electrophoretic motility of the partially melted doubled-stranded DNA molecule that will stop its migration in the gel, according to its melting behavior, and form a band in the gel. The number of bands or phylotypes or operational taxonomic units (OTU) will be representative of microbial diversity in environmental samples (Muyzer and Smalla, 1998). Comparisons between the molecular approach and the traditional method of conidial identification showed that the latter appears to underestimate fungal diversity on leaves. The molecular approach also allows us to estimate fungal diversity independently of the presence of reproductive structures, but its ability to distinguish among major fungal groups is still limited (Nikolcheva *et al.,* 2003; reviewed in Duarte *et al.,* 2012). In addition, the relative intensities of each band (phylotypes or OTUs) on DGGE gels might provide a semi-quantitative estimate of the relative biomass of species within the fungal communities (Nikolcheva and Bärlocher, 2005; Pascoal *et al.,* 2010).

However, in DGGE, different sequences can co-migrate to the same position of the gel, whose homogeneity cannot be established without further analyses, e.g. by extracting and sequencing the DNA from an individual DGGE band. In spite of its limitations, the DGGE technique proved to be a fast and valuable tool in detecting shifts in fungal communities either along time of leaf decomposition in streams (Duarte *et al.,* 2010; Nikolcheva *et al.*, 2003, 2005) or when exposed to several environmental stressors (e.g. Duarte *et al.,* 2009; Moreirinha *et al.*, 2011; Pradhan *et al.,* 2011).

More recently, by using other molecular techniques, such as cloning libraries, Seena and collaborators (2008) were able to recover fungal sequences from plant-litter decomposing in freshwaters. However, due to the limited number of reference sequences in DNA databases at that time, a low number of OTUs were connected to aquatic fungal species. As other molecular techniques, cloning libraries are also based on the extraction of whole-community DNA, followed by amplification with fungal-specific primers and the establishment of ribosomal gene libraries (Schadt *et al.,* 2003; Vandenkoornhuyse *et al.,* 2002). The phylogenetic analyses of randomly cloned sequences, allows estimations of the contribution of various fungal groups to the community DNA pool based on the frequencies of occurrence. This approach allows, theoretically, much greater resolution and unequivocal assignment to a taxon then other fingerprinting techniques such as DGGE (Seena *et al.,* 2008).

Numerous targets within the fungal genome have been evaluated to be used to detect fungal species in environmental samples and the most suitable ones are within the ribosomal DNA (rDNA) gene complex (Iwen *et al.,* 2002). This part of the genome includes the 18S, 5.8S and 28S genes, which code for ribosomal RNA (rRNA) that have relatively conserved nucleotide sequences among fungal species. On the other hand, the internal transcribed spacer region (ITS) is a non-coding region and was recently declared as the most suitable barcode to be used for the identification of fungal species (Schoch *et al.,* 2012) including those from environmental samples (Nilsson *et al.,* 2009).

In a near future, a better knowledge of the fungal diversity on decomposing plant-litter in streams might be provided by next-generation sequencing techniques, such as pyrosequencing (Bärlocher, 2010; Duarte *et al.,* 2013, Nilsson *et al.,* 2009). Pyrosequencing offers platforms of directly recovering all genetic material present in an environmental sample avoiding the tedious detour over clone libraries (DeLong, 2009; Edwards *et al.,* 2006) and is revolutionizing how the complexity of microbial communities is described and compared (Amend *et al.,* 2010).

This technique enables a rapid characterization of microbial communities in shorter time and at greater sequence depth than via cloning or with Sanger sequencing (Sogin *et al.,* 2006) and will allow the characterization of much larger numbers of sequences than those that are presently known (Bärlocher, 2010). Several pyrosequencing projects were already successfully conducted in order to analyse fungal diversity in environmental samples, such as the case of soils (e.g. Buée *et al.,* 2009).

1.4. Effects of eutrophication on plant-litter decomposition and associated biota

Ecosystems structure and functioning are being strongly altered by human activities (Dudgeon *et al.,* 2006; Vitousek *et al.,* 1997). Within ecosystems, freshwaters are among the most endangered ones and those with higher declines in biodiversity comparing to the most affected terrestrial systems (Sala *et al.,* 2000). Major threats to global freshwater biodiversity are: overexploitation, water pollution, alterations in flow regimes, destruction or degradation of the aquatic habitat and the invasion by exotic species that are resulting in several declines of freshwater biodiversity worldwide (Allan and Castillo, 2007; Dudgeon *et al.,* 2006).

The degradation of water resources has being increasing and, consequently, many industrialized countries are trying to improve the quality of the water resources and to restore, as much as possible, the ecological integrity of streams. In this context, it is important to implement new biological monitoring approaches to assess structural and functional integrity (Gessner and Chauvet, 2002; Pascoal *et al.,* 2003). Ecological integrity can be expressed as "the capability of supporting and maintaining a balanced, integrated, adaptative community of organisms having a species composition, diversity, and a functional organization, comparable to that of natural habitat of the region" (Cairns, 1975; Karr and Dudley, 1981). According to Gessner and Chauvet (2002), structural integrity can be defined in terms of quantitative and qualitative community composition and resources, whilst functional integrity is referred to rates, patterns and the relative importance of different ecosystem processes.

In the past, the assessment of human impacts on freshwater ecosystems was based only on physical and chemical characteristics of the stream water (Karr, 1991). Nowadays, the ecological integrity of freshwater ecosystems mainly relies on structural parameters of aquatic communities (Barbour *et al.,* 1999) but, to assess ecological integrity, both structural and functional components must be considered (Gessner and Chauvet, 2002; Pascoal *et al.,* 2001, 2003).

Leaf litter is the major source of nutrients and energy in low-order streams (Fisher and Likens, 1973; Pascoal *et al.,* 2005b). Plant-litter decomposition is considered an integrative net process that links riparian vegetation, microbial and invertebrate activities, and the physical and chemical environment of the stream (Benfield, 1996; Gessner *et al.,* 1999; Pascoal *et al.,* 2003). In addition, plant-litter decomposition is a key ecosystem process that responds to water quality (Pascoal *et al.,* 2001) and it was proposed as an effective measure to assess the integrity of

freshwater ecosystems (Gessner and Chauvet, 2002; Pascoal *et al.,* 2003). The response and role of microorganisms and invertebrates in polluted streams varies according to the type of stress (Metcalfe-Smith, 2009). It is known that species within macroinvertebrate communities have different sensitivities to various types of pollutants (Metcalfe-Smith, 2009; Pascoal *et al.,* 2003) and this is the main reason why they have been used to assess the ecological quality of freshwaters for decades (Lecerf, 2006; Rosenberg and Resh, 1993; Wright *et al.,* 2000).

Urbanization and agriculture runoff are considered to be the major sources of nitrogen and phosphorus in streams (Allan and Castilo, 2007). High nutrient loads in streams (eutrophication) are reported to affect ecosystem functions, such as plant-litter decomposition (Del Arco *et al.,* 2012; Duarte *et al.,* 2009; Pascoal and Cássio, 2004) and may also change diversity, composition and activity of aquatic communities, leading to changes in the detritus food webs (Pascoal *et al.,* 2005a,b). For instance, nutrient enrichment can stimulates plant-litter decomposition in streams (Duarte *et al.,* 2009; Grattan and Suberkrop 2001; Pascoal and Cássio, 2004). High concentrations of inorganic nutrients, as nitrogen and phosphorus, are reported to stimulate fungal activity as reproduction and productivity, leading to the enhancement of organic matter turnover (Ferreira *et al.,* 2006; Pascoal *et al.,* 2003, 2005a, b; Sridhar *et al.,* 2009).

Moreover, the experimental addition of these nutrients to stream water enhanced aquatic hyphomycete diversity in the water column (Gulis and Suberkroop, 2004) and fungal activity on decomposing leaves (Gulis and Suberkroop, 2003; Pascoal *et al.,* 2005a). But, in some cases when nitrogen or phosphorus is not limiting, the increase of their concentration in the stream water may not stimulate litter decomposition or activity of the associated microorganisms (Grattan and Suberkroop, 2001; Royer and Minshall, 2001; Ferreira *et al.,* 2006). In eutrophic streams, the low concentration of dissolved oxygen and high sedimentation rates can lead to the depression of fungal activity and litter decomposition (Pascoal and Cássio, 2004; Pascoal *et al.,* 2005a, b). Thus, the interaction with other stream variables can counteract the stimulating effects that nutrient enrichment have on aquatic biota (Pascoal and Cássio, 2004; Duarte *et al.,* 2009).

1.5. Aim of the thesis

Plant-litter decomposition in streams represents a key ecosystem process that connects riparian vegetation, microbial and invertebrate activities and environmental factors (Gessner *et al.,* 2009). Microorganisms, in particular aquatic fungi, play an important role in food webs by mineralizing organic matter and transforming it into a more palatable food source for macroinvertebrate detritivores, allowing the transference of carbon and energy along detritus-foodwebs (Bärlocher, 2005). However, these interactions can be strongly affected by anthropogenic stressors, in particular eutrophication, which is quite common in streams of Northwest Portugal, due to intensive industrial and agricultural activities (Duarte *et al.,* 2008, 2009; Mesquita *et al.* 2007; Pascoal and Cássio, 2004; Pascoal *et al.,* 2005a, c; Shridar *et al.,* 2009).

Although macroinvertebrate communities have been extensively used to assess the ecological integrity of freshwaters, in particular the effects of eutrophication (e.g. Castela *et al.,* 2008), an integrative approach including functional components, such as plant-litter decomposition has been claimed (Gessner and Chauvet 2002; Pascoal *et al.*, 2001, 2003, 2005a, b). In addition, aquatic fungi are in the basis of detritus food webs and previous studies indicated that they are sensitive to alterations in water quality (Pascoal *et al.*, 2005c; Castela *et al.,* 2008, Duarte *et al.,* 2009). Thus, a more comprehensive picture of the environmental condition of a stream can be attained if we include the responses of fungal communities in the assessment. However, until date, few studies have included decomposer fungal communities of plant-litter in freshwaters to analyze its integrity (but see Pascoal *et al.*, 2003; Castela *et al.,* 2008; Geraldes, 2011). This might be due to the restricted number of available techniques to analyze the diversity of aquatic fungi; most studies relied only on the microscopic identification of the reproductive structures that can miss species that are not sporulating. An alternative to surpass this disadvantage is to assess fungal diversity by using molecular techniques (e.g. Duarte *et al.,* 2009).

The aim of this study was to assess the effects of eutrophication on fungal and macroinvertebrate communities of plant-litter decomposing in streams of Northwest Portugal. In addition, fungal diversity was assessed by using both traditional (analysis of the reproductive structures) and molecular techniques (DGGE), to surpass the limitations of the traditional techniques when assessing fungal diversity. Oak leaves were used and placed in coarse mesh-

bags to allow fungal and invertebrate colonization and immersed in 5 selected streams along a gradient of eutrophication in the Ave River basin. Physical and chemical parameters of the stream water were analysed by standard methods. The experiment was conducted during 43 days in autumn/winter 2012 and sampling was done after 9, 23 and 43 days of leaf immersion in the 5 streams.

2. Materials and methods

2.1. Study area

The Ave River is 98 Km long and is located in the Northwest of Portugal. Its basin covers about 1388 Km^2 and its source is in the Cabreira Mountain and flows in an east-west direction into the Atlantic Ocean (Araújo *et al.,* 1998; Soares *et al.,* 1999). The most important tributaries of the Ave River are the Este River (51 Km length, 245 Km^2 draining area), on the right bank, and the Vizela River (47 Km length, 240 Km^2 draining area), on the left bank (Soares *et al.,* 1999). Although the river water is used for agricultural and industrial purposes in this region, it is highly contaminated due to several industrial discharges into the river (Soares *et al.,* 1999). Water problems are related to the high industrial density in the middle and lower parts of the river basin which includes leather tanning, rubber manufacture and plastic production (Soares *et al.,* 2009). However, pollution control programs have been implemented in order to decrease e.g. metal contamination (Alves *et al.,* 2009), but not eutrophication, at least to our knowledge.

Five sampling sites, belonging to the Ave River basin, were selected: Couros, Selho, Andorinhas, Oliveira and Agra. Stream order varied from three to four being Couros and Selho streams of order four and the remaining streams of order three. Couros and Selho streams are located nearby the city of Guimarães in an area with high population density and agricultural and industrial activities. Andorinhas and Oliveira streams are located near small human settlements with some agricultural activities, in Póvoa de Lanhoso, and Agra stream is located in the Cabreira Mountain where human presence and impact is almost absent, at least at first sight, near Vieira do Minho. At the sampling sites few exotic tree species were found and the surrounding riparian vegetation consisted mainly of alder (*Alnus glutinosa* Gaertn.), oak (*Quercus robur* L.), black poplar (*Populus nigra* L.) and chestnut (*Castanea sativa* Miller).

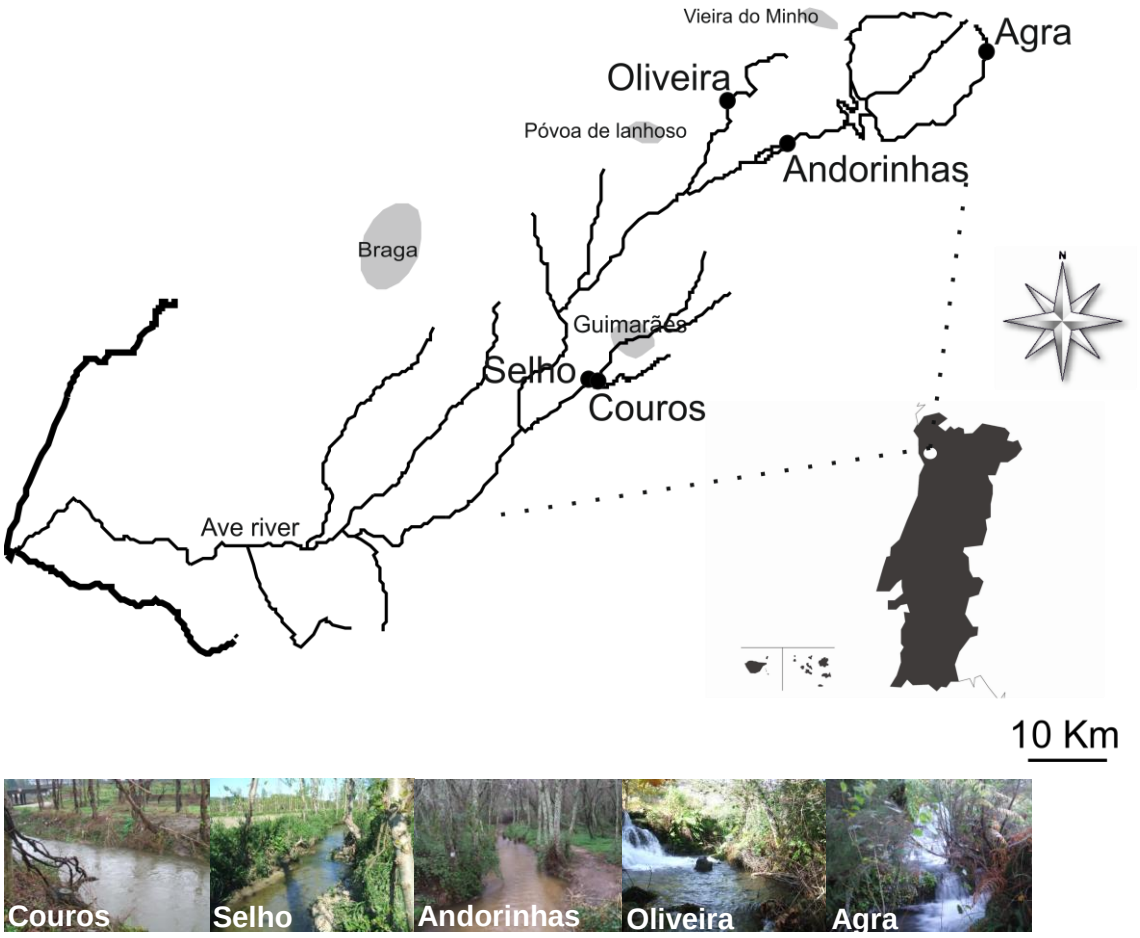

Figure 1 - Sampling sites location and main towns nearby, along the Ave River basin, in the Northwest of Portugal.

2.2. Leaf bags

Oak leaves were weighed into 3-g groups and placed, individually, into coarse-mesh bags (50 mm mesh size). A total of 80 bags were sealed and groups of 16 bags were distributed by each sampling site. This study started on 5th November 2012 and ran over 43 days with sampling dates after 9, 23 and 43 days of leaf immersion. On each sampling date, 4 replicate bags from each site were collected and transported to the laboratory for further analyses.

2.3. Physical and chemical analyses of stream water

On each sampling date, physical and chemical parameters of stream water were measured at each site. Temperature, pH, dissolved oxygen and conductivity were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW). Stream water samples were collected into plastic bottles and transported in a cool box (4°C) and water analyses were performed within 24 hours. Concentration of nitrates, nitrites, phosphates and ammonia were measured with HACH DR/2000 spectrophotometer (Hach Company, Loveland, CO, USA). Nitrate concentrations were quantified by the cadmium reduction method (measurable range: 0-0.4 mg L^{-1} and 0-30 mg L^{-1} $N\text{-}NO_3^-$), ammonia concentrations by the salicylate method (measurable range: 0-0.5 mg L^{-1} $N\text{-}NH_3$), nitrite concentrations by the diazotization method (measurable range: 0-0.3 mg L^{-1} $N\text{-}NO_2^-$) and phosphate concentrations by the ascorbic acid method (measurable range: 0-2.5 mg L^{-1} PO_4^{3-}), according to the HACH protocols manual.

2.4. Leaf mass loss

The leaves, collected from the mesh bags on each sampling date, were rinsed with deionized water to remove the sediments and adhering invertebrates. Leaf disks, with 12-mm diameter, were cut and two sets of 8 were freeze-dried and 10 leaf disks were used to induce fungal sporulation. Remaining leaves were dried at 80 °C for 48 hours in an oven (Memmert) and weighed to the nearest 0.0001 g (Mettler Toledo). Leaf mass loss was expressed in degree days, which were calculated by multiplying the time in days by the average temperature measured at each stream during the experiment.

2.5. Fungal sporulation and biomass

Fungal sporulation was induced by aeration of 10 leaf discs from each bag, in 75 mL filtered stream water (0.22 pore diameter, Sarstedt) for 48 hours at 16°C. Conidial suspensions were collected into plastic bottles and 4 mL of 37% of formaldehyde and 100 µL of 15% Triton X-100 were added in order to stop sporulation and avoid conidia to adhere to the bottles, respectively. Appropriate volumes of each replicate were filtered through membranes with 0.45

µm pore size (Millipore). Half of each membrane was placed between a glass slide and a cover slip and spores on the membranes were stained with cotton blue in lactic acid (Fluka), before counted and identified under a light microscope with a magnification of 400x (Leica BioMed). Sporulation rates were calculated as the number of conidia released per gram of leaf dry mass per day.

Fungal biomass on leaves was estimated from ergosterol concentration accordingly to the protocol of Gessner (2005). Briefly, sets of 8 leaf disks were placed in extraction tubes containing a solution of KOH/methanol (8 g L^{-1}) and were heated for 30 min at 80 °C, in order to perform lipid extraction. Ergosterol was then purified by solid phase extraction (Sep-Pak, Waters) and eluted to high performance liquid chromatography (HPLC) vials with isopropanol (Fluka). Ergosterol was quantified by HPLC in a Thermo Scientific Dionex UltiMate 3000 Series equipment (Thermo Scientific) and using a RP18 Lichrospher column with 250 mm length and 4.6 mm internal diameter (Merck). The elution of ergosterol from the column was performed with methanol HPLC grade (Fisher Scientific) with a flow rate of 1.4 mL min^{-1} and ergosterol peaks were detected at 282 nm. Ergosterol was converted to fungal biomass by using a factor of 5.5 µg ergosterol mg^{-1} fungal dry mass (Gessner and Chauvet, 1993).

2.6. Macroinvertebrate sampling

On each sampling date, benthic macroinvertebrates were collected from the mesh bags. Macroinvertebrates were then transferred to 50 mL falcon tubes, preserved in 96% ethanol, for further counting and identification. Macroinvertebrates were identified to the lowest possible taxonomic level, accordingly to Tachet *et al.*, (2010) using a stereo microscope (Leica). Invertebrates were assigned to shredders according to Merritt and Cummins (1996). After identification, invertebrates were separated into shredders and non-shredders and dried at 80°C for 48 hours and weighed to the nearest 0.0001 g, in order to determine invertebrates' biomass.

2.7. Molecular analyses

DNA was extracted from four freeze-dried leaf disks using a soil DNA extraction kit (MoBio Laboratories), accordingly to the manufacturer's instructions. The ITS2 region of fungal ribosomal

DNA was amplified using the primers ITS3GC and ITS4 (White *et al.,* 1990). For PCR, 12.5 µL of GoTaq Mix (Promega) was used, plus 10.5 µL of Ultra-pure water, 0.5 µL of each primer and 1 µL of DNA, in a final volume of 25 µL. Fungal PCRs were carried out in a VWR Thermal Cycler DOPPIO (VWR International) using the following program: initial denaturation at 95°C for 2 minutes, followed by 36 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72°C for 1 minute. The final extension was done at 72°C for 5 minutes (Duarte *et al.,* 2009; Nikolcheva *et al.,* 2005).

DGGE analyses were performed using a DCode universal mutation detection system (Bio-Rad laboratories). Samples of 15-40 µL of fungal DNA were loaded on an 8 % (w/v) polyacrilamide gel in 1x Tris-acetate-EDTA (TAE) buffer with a denaturing gradient of formamide and urea from 30 to 60% (100% denaturant corresponds to 40% formamide and 7 M urea). The gels were run at 55 V and 56°C for 16 hours and stained with 100 mL of Midori Green 1x (Nipon Genetics) for 10 minutes. The gel images were captured under UV light in a gel documentation system GenoSmart (VWR, International).

A marker was prepared by mixing equal amounts of DNA of 8 aquatic hyphomycete strains: *Alatospora pulchella* UMB-1115.13, *Anguillospora filiformis* UMB-1088.13, *Articulospora tetracladia* UMB-1127.13 and UMB-1136.13, *Dimorphospora foliicola* UMB-1085.13, *Tricladium chaetocladium* UMB-1101.13, *T. splendens* UMB-1117.13 and *Triscelophorus* cf. *acuminatus* UMB-1118.13.

2.8. Data analyses

Kolmogorov-Smirnov tests were used to test if data followed a normal distribution. Data from stream water parameters were compared between streams using one-way ANOVAs (Zar, 2009). Tukey's post tests were used to assess where significant differences occurred (Zar, 2009). A Principal Component Analysis (PCA) was used to ordinate streams according to physical and chemical variables of the stream water. Normalization of the variables was done prior to the PCA analysis (Legendre and Legendre, 1998).

Values of leaf dry mass remaining of oak leaves were fit to the exponential model $m_t = m_0 \times e^{-kt}$, where m_t is leaf dry mass remaining at time t, m_0 is the initial leaf dry mass and k is the rate of leaf decomposition. Since temperature was significantly different between streams, degree

days were used to normalize for the direct effect of temperature on decomposition rates. Degree days were calculated as time in days multiplied by the average temperature during the experiment for each stream. Regression lines of ln-transformed values of leaf dry mass remaining were compared by analysis of covariance (ANCOVA) (Zar, 2009).

DGGE gels were aligned and normalized in GelCompar II. Each DGGE band was considered to be an operational taxonomic unit (OTU) because the DNA from more than one species can co-migrate to the same position in the gel.

Two-way ANOVAs were used to test if the time, stream and the interaction between both factors (time x stream) significantly affected fungal sporulation rates, fungal biomass, fungal diversity, assessed from fungal spores or DGGE OTUs, invertebrate diversity and biomass (Zar, 2009). Tukey's post tests were used to assess where these differences occurred (Zar, 2009).

Shannon's diversity (*H'*) and Pielou's equitability (*J'*) indices were used to assess the diversity of aquatic fungi and invertebrates as follows:

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

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

Where *Pi* is the relative abundance of taxon *i* and S is the total number of taxa. Ordination of between fungal and invertebrate communities by sampling time and streams, were assessed by non-metric multidimensional scaling (Kruskal and Wish, 1978) based on the Bray-Curtis similarity index. Overlay clusters representing a resemblance level of 40%, 64% and 30% were superimposed in the MDS diagrams of fungal communities, assessed as fungal spores and OTUs, and invertebrate communities, respectively. ANOSIM was used to test for differences in community structure between streams and sampling times and using a maximum of 999 permutations (Clarke and Gorley, 2006). The ANOSIM test statistic R varies between 0 and 1, with 0 indicating absence of differences and 1 corresponding to maximum dissimilarity between communities (Clarke and Gorley, 2006).

Multivariate analyses (PCA and MDS) were performed with PRIMER 6 (Primer-E, UK) for Windows. ANOVAs were performed using STATISTICA 8 (StatSoft, USA) and ANCOVAs using GraphPad Prism for Windows (GraphPad software Inc., San Diego). GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used for processing the DGGE gels and to assess the relative intensities of bands in the DNA fingerprints.

3. Results

3.1. Physical and chemical characteristics of the stream water

Physical and chemical parameters of the stream water in the 5 study streams are presented in Table 1. Values of pH varied slightly between the streams (6.4 and 7.0 for Agra and Oliveira, respectively), and differences were not significant (1-way ANOVA, P=0.4) (Table 1). Stream water temperature ranged from 8.4 to 12.6°C increased by the following order: Agra < Oliveira < Andorinhas < Selho < Couros (Table 1) and significantly differed between streams (1-way ANOVA, P<0.05; Tukey's post tests, P<0.001). Conductivity was significantly different between streams (1-way ANOVA, P<0.001) and values ranged from 15.8 to 236.4 μS cm^{-1} in Agra and Couros streams, respectively (Table 1). Conductivity was significantly higher in the Couros stream than in Andorinhas, Oliveira and Agra streams (Tukey's post tests, P<0.01).

The Oliveira stream had the highest dissolved oxygen (11.3 mg L^{-1}), while Couros had the lowest one (7.7 mg L^{-1}) (Table 1). Dissolved oxygen was lower in Couros than in Agra, Oliveira and Selho streams (Tukey's post tests, P<0.05). Current velocity ranged from 12.6 to 36.6 Km h^{-1} in Selho and Agra streams, respectively (Table 1) and this difference was significant (1-way ANOVA, P=0.002; Tukey's post tests, P<0.05).

Concentrations of phosphates, nitrates, nitrites and ammonia also differed between streams (1-way ANOVA, P<0.001). The Couros stream presented significantly higher levels of phosphates (684.0 μg L^{-1} of PO_4^{3-}), nitrites (145.6 μg $L^{-1}$of $N\text{-}NO_2^-$) and ammonia (2,344 μg L^{-1} of $N\text{-}NH_3$) than those found at the remaining streams (Tukey's post tests, P<0.05), while Agra presented the lowest ones (16.0 μg L^{-1} of PO_4^{3-}, 4.6 μg L^{-1} of $N\text{-}NO_2^-$ and 6.0 μg L^{-1} of $N\text{-}NH_3$) (Table 1). Nitrate concentrations varied between 64.0 and 2,240 μg L^{-1} of $N\text{-}NO_3^-$ for Agra and Selho streams, respectively (Table 1). Significantly higher values of nitrates were also found in Couros and Selho than in the other streams (Tukey's post tests, P<0.05).

Table 1 - Physical and chemical characteristics of the stream water in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). Data represent mean values ± SEM (n=5), except for Temperature (n=120) and current velocity (*n=3;**n=2).

	Stream				
	Couros	**Selho**	**Andorinhas**	**Oliveira**	**Agra**
Longitude N (^{0})	41.43741	41.43809	41.56979	41.5863	41.60979
Latitude W (^{0})	8.32175	8.32253	8.17704	8.22513	8.03883
Elevation (m)	149	149	210	232	776
pH	7.0 ± 0.2	7.0 ± 0.4	6.9 ± 0.6	7.0 ± 0.6	6.5 ± 0.6
Conductivity ($\mu S\ cm^{-1}$)	236.4 ± 101.7	127.6 ± 49.4	84.4 ± 71.4	58.4 ± 43.9	15.8 ± 0.4
Current velocity ($Km\ h^{-1}$)	25.2 ± 2.8*	12.6 ± 9.6*	28.9 ± 10*	23.6 ± 11.9*	36.6 ± 24.5**
Oxygen ($mg\ L^{-1}$)	7.7 ± 1.1	11.0 ± 1.2	10.5 ± 2.5	11.3 ± 1.5	11.0 ± 1.6
Saturation (%)	73.2 ± 13.4	99.9 ± 16.6	98.3 ± 21.5	103.1 ± 13.9	106.5 ±13.4
Temperature (ºC)#	12.6 ± 4.7	11.3 ± 5.5	10.8 ± 4.5	10.4 ± 5.1	8.4 ± 4.7
Phosphates ($\mu g\ L^{-1}\ PO_4^{3-}$)	684.0 ± 214.0	272.0 ± 157.0	58.0 ± 36.0	34.0 ± 18.0	16.0 ± 13.0
Nitrates ($\mu g\ L^{-1}\ N\text{-}NO_3^-$)	1,600 ± 579.0	2,240 ± 428.0	940.0 ± 241.0	520.0 ± 148.0	64.0 ± 9.0
Nitrites ($\mu g\ L^{-1}\ N\text{-}NO_2^-$)	145.6 ± 68.0	37.2 ± 25.0	6.2 ± 2.0	5.6 ± 3.0	4.6 ± 1.0
Ammonia ($\mu g\ L^{-1}\ N\text{-}NH_3$)	2,344 ± 1,420	544.0 ± 430.0	10.0 ± 10.0	6.0 ± 5.0	6.0 ± 5.0

The PCA ordination of the 5 streams according to the stream water variables showed that axis 1 explained 73.5% of the total variance and axis 2 explained 20.8% of the total variance (Figure 2). PC1 separated streams according to differences in conductivity and the levels of phosphates, nitrites and ammonia, while PC2 separated streams, according to current velocity, pH and the levels of nitrates in the stream water. The PCA clearly indicated a gradient of eutrophication from Agra, the most oligotrophic stream, Andorinhas and Oliveira, moderately eutrophic streams, to Selho and Couros, the most eutrophic streams (Figure 2).

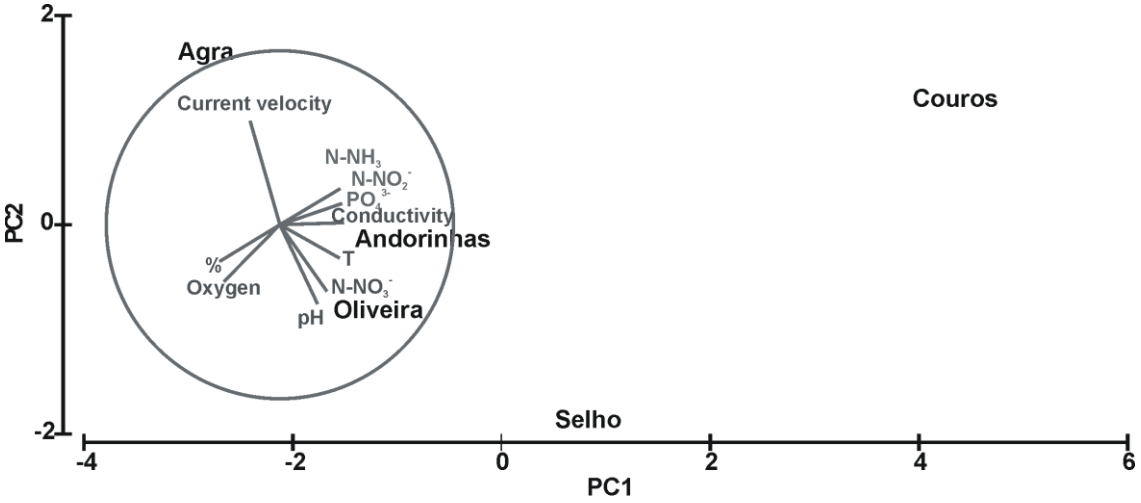

Figure 2 - Principal Component Analysis (PCA) of the physical and chemical characteristics of the water in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra).

3.2. Leaf decomposition rates

Decomposition rates of oak leaves immersed in the 5 streams are presented in Table 2. Decomposition rates varied between 0.0002 and 0.0023 degree days^{-1} in Couros, the most eutrophic stream, and in Oliveira, a moderately eutrophic stream, respectively. Decomposition rates differed significantly between streams (ANCOVA, $P<0.05$) and increased by the following order: Couros < Selho < Agra < Andorinhas < Oliveira (Table 2).

Tabela 2 - Decomposition rates (k) of oak leaves immersed in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra).

Stream	k (degrees day^{-1}) ± SE	W_o (%)	r^2
Couros	-0.00020 ± 0.000065^{a}	95.5	0.54
Selho	-0.00058 ± 0.000093^{a}	94.9	0.73
Andorinhas	-0.0014 ± 0.000039^{b}	99.9	0.99
Oliveira	-0.0023 ± 0.00028^{c}	106.6	0.82
Agra	-0.0011 ± 0.000074^{a,b}	96.3	0.93

W_o, Intercept; r^2- coefficient of determination. Identical letters indicate no significant differences between streams (Tukey's post tests).

Decomposition rates of oak leaves were significantly lower in Couros and Selho streams, the most eutrophic streams, than at Andorinhas and Oliveira streams (Tukey's post tests, $P<0.05$), the two moderately eutrophic streams, but did not differ from leaves decomposing at Agra, the most oligotrophic stream (Tukey's post test, $P>0.05$). In addition, leaf decomposition rates did not differ between the two hypertrophic streams, Couros and Selho (Tukey's post test, $P>0.05$), but differed between the two moderately eutrophic streams Oliveira and Andorinhas, with Oliveira presenting higher values than Andorinhas (Tukey's post test, $P<0.05$). No differences were found between oak leaves decomposing in Andorinhas and Agra streams, but decomposition rates were significantly higher in Oliveira than in Agra stream (Tukey's post tests, $P<0.01$).

3.3. Fungal sporulation rates

Fungal sporulation rates on decomposing oak leaves varied in average between 8.8 x 10^4 and 1.1 x 10^6 conidia g^{-1} dry mass d^{-1} in Couros and Oliveira streams, respectively, and increased by the following order: Couros < Agra < Selho < Andorinhas < Oliveira (Figure 3).

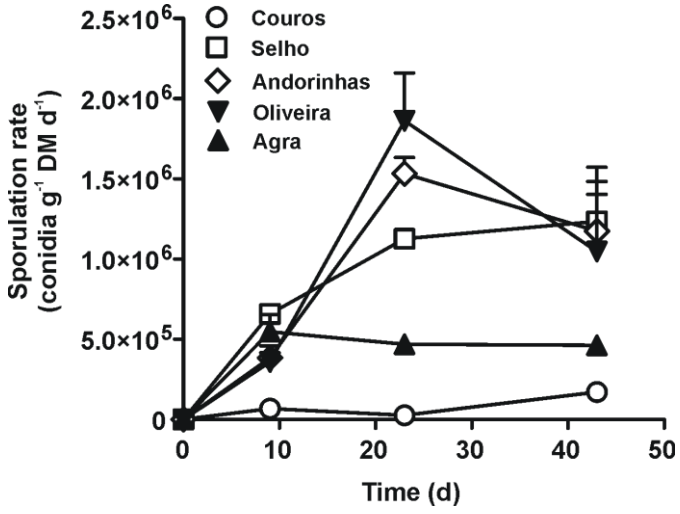

Figure 3 - Sporulation rates of aquatic fungi on oak leaves decomposing in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). Data represent means ± SEM (n=3).

Fungal sporulation rates peaked after 23 days at the two moderately eutrophic streams Oliveira and Andorinhas (1.9 x 10^6 and 1.5 x 10^6 conidia g^{-1} dry mass d^{-1}, respectively), while at Agra maximum values were attained after 9 days of stream immersion (5.5 x 10^5 conidia g^{-1} dry mass d^{-1}). On the other hand, at the most eutrophic streams Selho and Couros, sporulation rates attained maximum values only after 43 days of leaf immersion (1.2 x 10^6 and 1.6 x 10^5 conidia g^{-1} dry mass d^{-1}, respectively) (Figure 3).

Sporulation rates were significantly affected by stream, time and the interaction between both factors (2-way ANOVA, Table 3). Fungal sporulation rates on oak leaves were significantly lower in the Couros stream than in Selho, Andorinhas and Oliveira streams (Tukey's post tests, P = 0.0001, for all comparisons), but did not differ of those on leaves decomposing in Agra stream (Tukey's post test, P = 0.08). In addition, sporulation rates were significantly higher on leaves decomposing in Selho, Andorinhas and Oliveira than on leaves decomposing in the most oligotrophic stream Agra (Tukey's post tests, P≤0.01).

Table 3 - Two-way ANOVAs on the effects of the stream, time and the interaction between stream and time on fungal sporulation rates.

Treatment	SS	DF	MS	F	P
Stream	6.9 x 10^{12}	4	1.7 x 10^{12}	16.9	<0.000001
Time	2.8 x 10^{12}	2	1.4 x 10^{12}	13.9	0.00005
Stream x Time	3.2 x 10^{12}	8	4.0 x 10^{11}	4	0.002
Error	3.0 x 10^{12}	30	1.0 x 10^{11}		

3.4. Fungal biomass

Fungal biomass varied in average between 8.4 and 48.1 mg g^{-1} leaf dry mass in Couros and Andorinhas streams, respectively. Fungal biomass increased by the following order: Couros < Selho < Agra < Oliveira < Andorinhas (Figure 4).

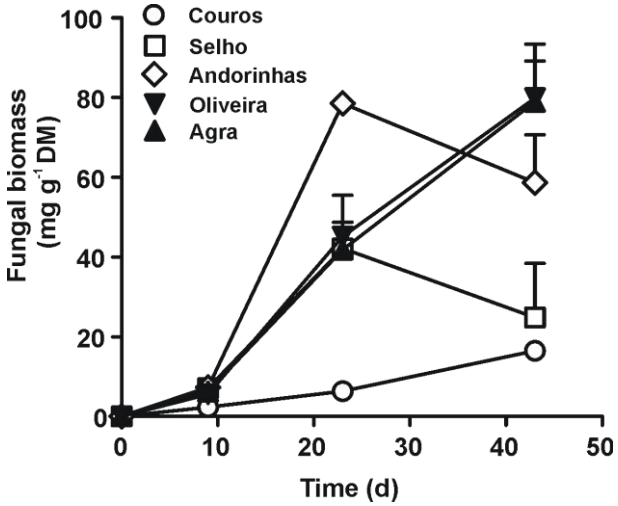

Figure 4 - Fungal biomass on oak leaves decomposing at the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). Data represents means ± SEM (n=3).

Maximum values of fungal biomass were attained after 23 days on oak leaves decomposing in Selho (42.1 mg g^{-1} leaf dry mass) and Andorinhas (78.5 mg g^{-1} leaf dry mass), and after 43 days on oak leaves decomposing at Couros, Oliveira and Agra streams (16.5, 79.8 and 78.9 mg g^{-1} leaf dry mass, respectively) (Figure 4).

Fungal biomass on oak leaves was significantly affected by the stream, time and interaction between both factors (2-way ANOVA, Table 4). Significantly lower fungal biomass was found on oak leaves decomposing in the most eutrophic streams (Couros and Selho) than on leaves decomposing at Andorinhas, Oliveira and Agra streams (Tukey's post-tests, $P \leq 0.0001$ and $P < 0.05$, respectively). No significant differences were found for fungal biomass between leaves decomposing in Andorinhas, Oliveira and Agra streams (Tukey's post-tests, $P > 0.05$).

Table 4 - Two-way ANOVAs on the effects of the stream, time and the interaction between stream and time on fungal biomass.

Treatment	SS	DF	MS	F	P
Stream	9908.9	4	2477.2	15.0	0.000001
Time	17852.8	2	8926.4	54.1	<0.000001
Stream x Time	8593. 9	8	1074.2	6.5	0.00006
Error	4948.3	30	164.9		

3.5. Invertebrates biomass

Invertebrate biomass varied in average between 2.2 and 80.4 mg g^{-1} leaf dry mass in, Oliveira and Selho streams, respectively, and increased by the following order: Oliveira < Agra < Andorinhas < Couros < Selho (Figure 5).

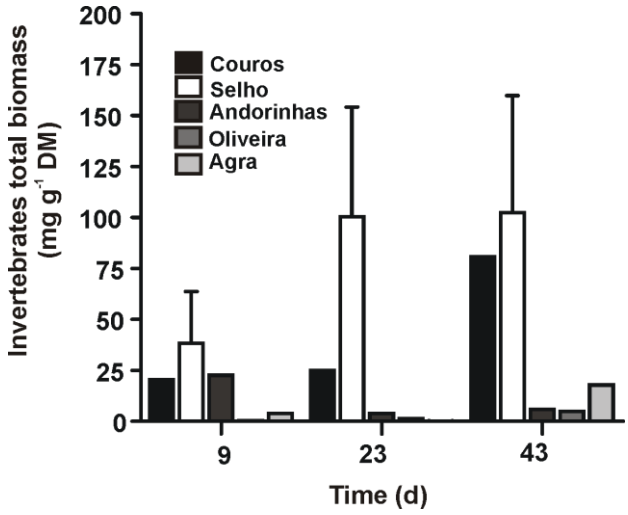

Figure 5 - Invertebrate biomass on oak leaves decomposing at the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). Data represents means ± SEM (n=3).

Maximum values of invertebrates' biomass were attained after 43 days on oak leaves decomposing in Selho (102.4 mg g^{-1} leaf dry mass), Couros (80.8 mg g^{-1} leaf dry mass), Oliveira (4.8 mg g^{-1} leaf dry mass) and Agra (17.9 mg g^{-1} leaf dry mass), and after 9 days in Andorinhas (22.6 mg g^{-1} leaf dry mass) (Figure 5).

Invertebrate biomass on oak leaves was significantly affected by the stream but not by the time or interaction between factors (2-way ANOVA, Table 5). Significantly higher values for invertebrate biomass were found on oak leaves in Selho, the most eutrophic stream than in the less eutrophic streams Andorinhas, Oliveira and Agra (Tukey's post-tests, $P<0.05$). No differences were found for invertebrate biomass between Andorinhas, Oliveira and Agra (Tukey's post-tests, $P>0.05$) and between the hypertrophic stream Couros and the highly eutrophic stream Selho (Tukey's post-tests, $P>0.05$).

Table 5 - Two-way ANOVAs on the effects of the stream, time and the interaction between stream and time on invertebrates' biomass.

Treatment	SS	DF	MS	F	P
Stream	38950.4	4	9737.6	5.6	0.002
Time	4903.5	2	2451.7	1.4	0.300
Stream x Time	11072.3	8	1384.0	0.8	0.600
Error	52355.9	30	1745.2		

The percentage of shredder and non-shredder invertebrates' biomass on decomposing oak leaves varied between streams (Figure 6). In Couros and Selho streams, non-shredder invertebrates were the major contributor to the total invertebrate biomass (66. to 100%) (Figure 6). In general, invertebrate shredders contributed more to the total invertebrate biomass on leaves decomposing in Agra, Oliveira and Andorinhas streams (Figure 6). In these streams, shredders biomass reached 100% after 9 and 23 days of leaf immersion in Oliveira; after 23 and 43 days in Agra and after 43 days in Andorinhas (Figure 6).

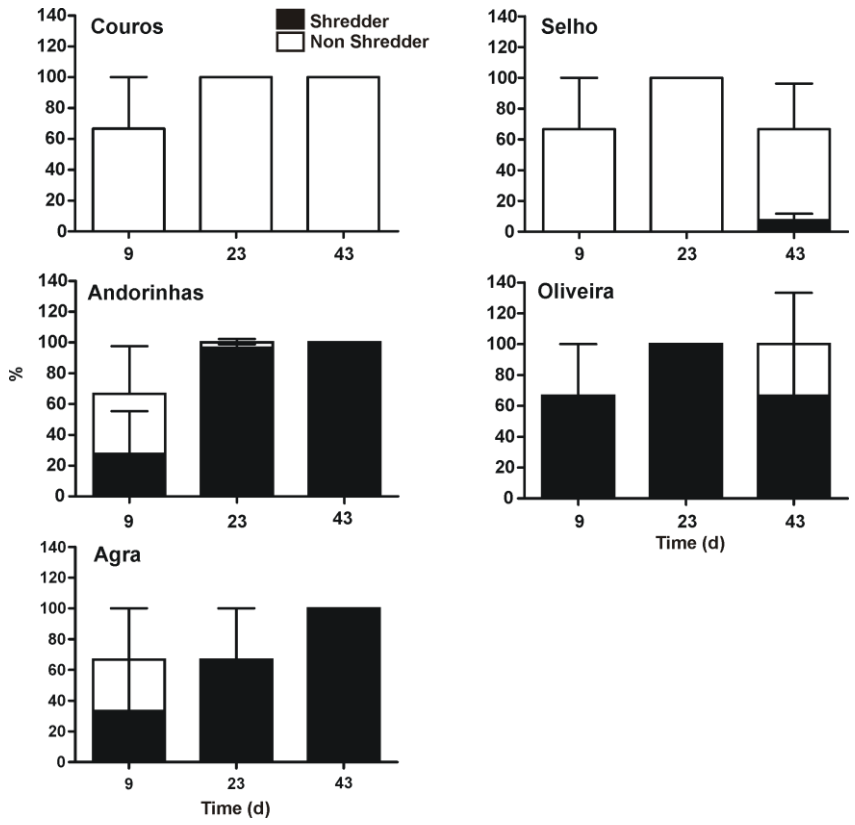

Figure 6 - Percentage contribution of invertebrate shredders and non-shredders for total invertebrates' biomass on oak leaves decomposing in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra).

3.6. Fungal diversity

A total of 19 aquatic fungal taxa were found sporulating on oak leaves in the 5 streams (Table 7). The Oliveira stream presented the highest richness in fungal taxa with 16 species sporulating on leaves, the highest Pielou's (*J'* from 0.6 to 0.8) and Shannon's diversity (*H'* from 0.9 to 1.7) indices, while the lowest values were found for leaves decomposing in the Couros stream (2 sporulating taxa, *J'* from 0.2 to 0.6, *H'* from 0.1 to 0.4, respectively).

Stream, time and interaction between both factors significantly affected the fungal sporulating taxon richness (2-way ANOVA) (Table 6). Fungal taxon richness was significantly higher in the moderately eutrophic streams Andorinhas and Oliveira than in the most eutrophic streams Couros and Selho and the most oligotrophic stream Agra (Tukey's post-tests, $P<0.05$). On the other hand, richness of sporulating fungal taxa did not differ between Andorinhas and Oliveira streams (Tukey's post tests, $P>0.05$).

Table 6 - Two-way ANOVAs on the effects of the stream site, time and interaction of stream and time on fungal diversity assessed from spores.

Treatment	SS	DF	MS	F	P
Stream	212.6	4	53.1	61.3	<0.000001
Time	19.5	2	9.8	11.3	0.000225
Stream x Time	60.5	8	7.6	8.7	0.000004
Error	26	30	0.9		

The contribution of fungal species to the total conidial production varied with the stream and time of leaf decomposition (Table 6). In the most eutrophic stream Couros, *Dimorphospora foliicola* was the dominant species, contributing with more than 84% to the total conidial production. This species was co-dominant (7.8 to 76.5%) with *Clavariopsis aquatica* (14.5 to 52.6%) on leaves decomposing in the highly eutrophic stream Selho (Table 7). *Tetrachaetum elegans* was dominant on oak leaves decomposing in the moderately eutrophic stream Andorinhas (29.9 to 68.3%) and Oliveira (20.2 to 65.3%). Other dominant species on leaves were *Clavatospora longibrachiata* (with up to 35%) in Andorinhas and *Articulospora tetracladia* (12.7 to 33.8%) in Oliveira (Table 7).

In the most oligotrophic stream Agra, *Articulospora tetracladia* was the dominant species (41.6 to 77.1%), and co-dominant taxa were *A. filiformis* (up to 18.1%), *L. curvula* (up to 18.4%) and *Fusarium* sp.(up to 37.4%) (Table 7).

Table 7 - Percentage contribution of each aquatic hyphomycete taxa to the total conidial production on oak leaves decomposing at the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). T1, T2 and T3 represent 9, 23 and 43 days of oak leaf immersion at each stream, respectively.

	Couros			Selho			Andorinhas			Oliveira			Agra		
Aquatic fungal taxa	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Alatospora acuminata Ingold								2.7			0.1				
A. pulchella Marvanová												2.7			
Anguillospora filiformis Greath.	15.9	4.9	4.9	3.5	8.5	14.1	1.9	0.3		2.5	4.9	2.8	17.8	18.1	
Articulospora tetracladia Ingold								1.8	2.1	22.5	33.8	12.7	74.2	77.1	41.6
Clavariopsis aquatica De Wild.				14.5	52.6	46.4		1.0	10.1						
Clavatospora longibrachiata (Ingold) Sv. Nilsson						0.6		1.9	35.0		0.5	3.2			
Culicidospora aquatica R.H. Petersen									3.9			0.1			
Dimorphospora foliicola Tubaki	84.1	95.1	95.1	76.5	14.4	7.8	11.5	0.8	0.4	3.0	21.5	7.3	3.7		2.5
Fusarium sp.															37.4
Goniopila monticola (Dyko) Marvanová & Descals							2.3	1.7	6.5		4.0	7.7			
Heliscella stellata (Ingold & VJ Cox)												1.1			
Heliscus lugdunensis Sacc. & Thérry				1.7			7.6	1.6		6.5	0.3				
Infundibura sp.				1.5			7.8				0.9	2.2			
Lunulospora curvula Ingold				2.4	6.2	28.3	0.5	10.9	4.3		5.2	0.3	1.1	2.9	18.4
Tetrachaetum elegans Ingold						0.5	68.3	65.2	29.9	65.3	20.3	20.2			
Tricladium chaetocladium Ingold					18.3	2.3		8.5	6.2	0.2	6.5	4.3	3.3		
T. splendens Ingold								3.6	1.7						
Triscelophorus cf. *acuminatus* Nawawi											2.0	34.9		1.9	0.1
T. monosporus Ingold												0.4			
Taxa richness	2.0	2.0	2.0	6.0	5.0	7.0	7.0	12	10	6.0	12.0	14.0	5.0	4.0	5.0
Pielou's evenness (*J'*)	0.6	0.2	0.2	0.4	0.8	0.7	0.6	0.5	0.7	0.6	0.8	0.8	0.5	0.5	0.7
Shannon index (*H'*)	0.4	0.1	0.1	0.8	1.3	1.3	1.0	1.2	1.4	0.9	1.7	1.7	0.8	0.7	1.0

Non-metric Multi-Dimensional Scaling (MDS) analysis of sporulating fungal communities on decomposing leaves indicated that the community structure differed between streams. Fungal communities were separated in four groups (overlay clusters) with 40% of similarity: 1) communities from the most eutrophic stream Couros; 2) communities from the high eutrophic stream Selho; 3) communities from the two moderately eutrophic streams Andorinhas and Oliveira; and 4) communities from the oligotrophic stream Agra (Figure 7).

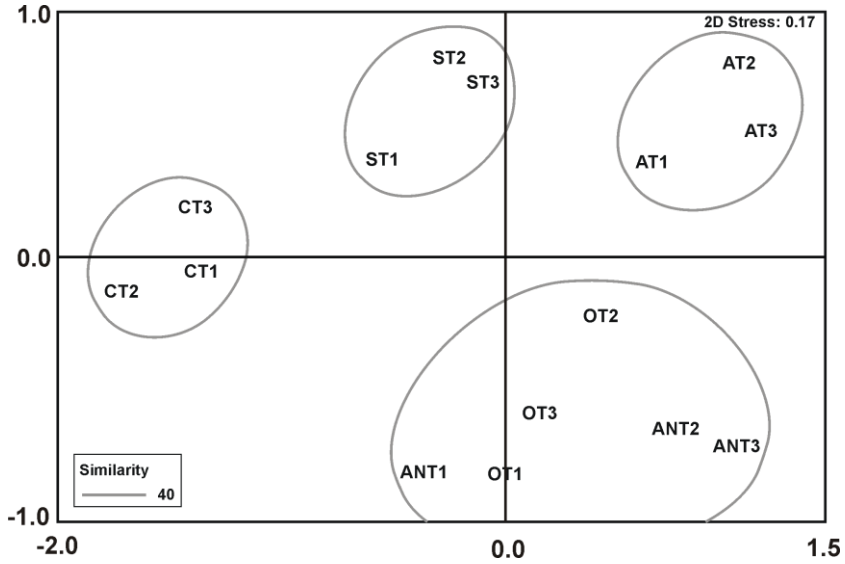

Figure 7 - Non-metric Multi-Dimensional Scaling (MDS) based on fungal sporulating communities on oak leaves decomposing in the 5 streams of the Ave River basin (C, Couros; S, Selho; AN, Andorinhas; O, Oliveira and A, Agra). T1, T2 and T3 represent 9, 23 and 43 days of leaf immersion in each stream, respectively.

Results were supported by ANOSIM tests that indicated that community structure significantly differ between streams (global R = 0.898). Pairwise tests indicated that maximum differences were found between communities on leaves decomposing in Agra *versus* Couros, Selho or Andorinhas and between communities on leaves decomposing in Oliveira and Couros (R = 1, for all comparisons). Strong differences in the structure of fungal communities based on sporulating taxa were also found between Selho *versus* Couros or Oliveira, and with Couros *versus* Andorinhas (R = 0.963, for all comparisons). Furthermore, community structure also differed but to lesser extent between leaves decomposing in Selho and Andorinhas (R = 0.889) and between Oliveira and Agra (R = 0.815). Community structure did not differ between Oliveira and Andorinhas streams (R = 0).

The average number of fungal operational taxonomic units (OTUs) from DGGE varied between 22.1 in Agra and 40.9 OTUs in Oliveira streams (Figure 8). The number of fungal OTUs increased by the following order: Agra < Andorinhas < Couros < Selho < Oliveira.

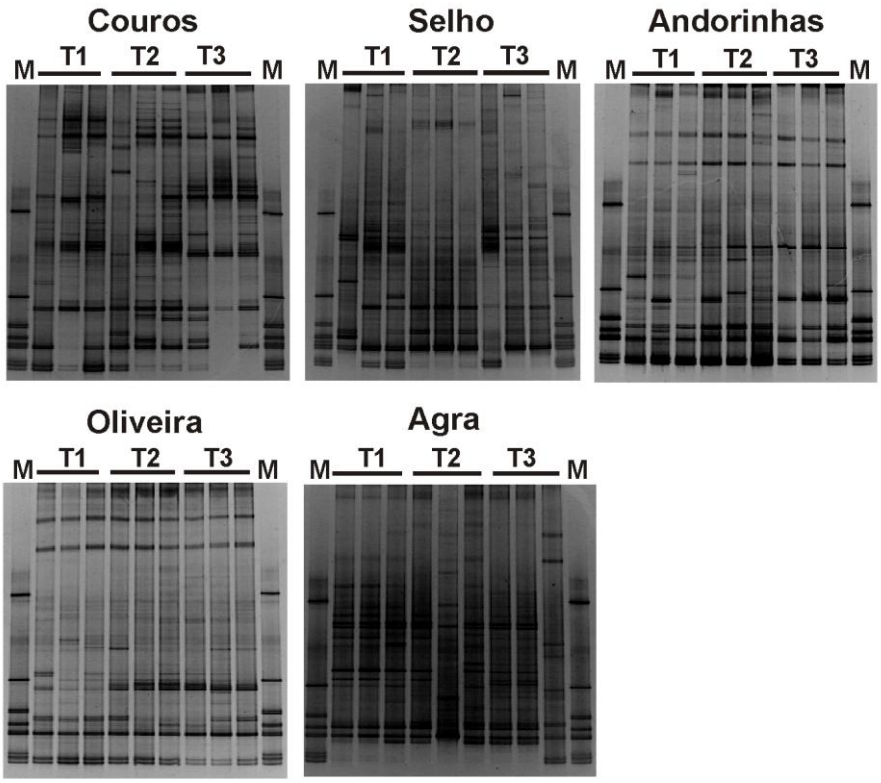

Figure 8 - DGGE fingerprints of fungal communities on oak leaves decomposing in the 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). M, DNA mixture of 7 strains of aquatic hyphomycete species (OTUs from top to down in each gel represent DNA from *Triscelophorus* cf. *acuminatus* UMB-1118.13, *Articulospora tetracladia* UMB-1136.13, *Alatospora pulchella* UMB-1115.13, *Articulospora tetracladia* UMB-1127.13, *Tricladium chaetocladium* UMB-1101.13, *Anguillospora filiformis* UMB-1088.13, *Tricladium splendens* UMB-1117.13 and *Dimorphospora foliicola* UMB-1085.13. T1, T2 and T3 represent 9, 23 and 43 days of oak leaf immersion in each stream, respectively.

Agra stream had the highest Pielou's (*J'* from 0.8 to 0.9) and Oliveira stream the highest Shannon's indices (*H'* from 3.0 to 3.1) while Andorinhas stream had the lowest Pielou's and Shannon's indices (*J'* from 0.7 to 0.8 and *H'* from 2.6 to 2.7, respectively)

The diversity of fungi assessed from DGGE OTUs was significantly affected by the stream but not by the time or interaction between stream and time (2-way ANOVA, Table 8).

Table 8 - Two-way ANOVAs on the effects of the stream site, time and interaction of stream and time on fungal diversity assessed from DGGE OTUs.

Treatment	SS	DF	MS	F	p
Stream	2053.6	1	513.4	21.3	<0.000001
Time	6.5	4	3.3	0.14	0.870
Stream x Time	386.4	2	48.3	2	0.080
Error	722.7	8	24.1		

The numbers of fungal OTUs were lower in the oligotrophic stream Agra than in the other streams (Tukey's post-tests, $P<0.01$). Furthermore, fungal OTUs on oak leaves were higher in Oliveira than in Andorinhas (Tukey's post-test, $P<0.05$).

Non-metric MDS ordination of fungal communities assessed from DGGE OTUs indicated that community structure differed between streams as shown by the presence of 6 groups (overlay clusters) with 64% of similarity in the ordination plot (Figure 9): 1) communities from the oligotrophic stream Agra; 2) communities from the highly eutrophic stream Selho; 3) communities from the most eutrophic stream Couros; 4) early communities (9 and 23 days) from the moderately eutrophic stream Andorinhas; 5) late communities (43 days) from the moderately eutrophic stream Andorinhas; and 6) communities from the moderately eutrophic stream Oliveira.

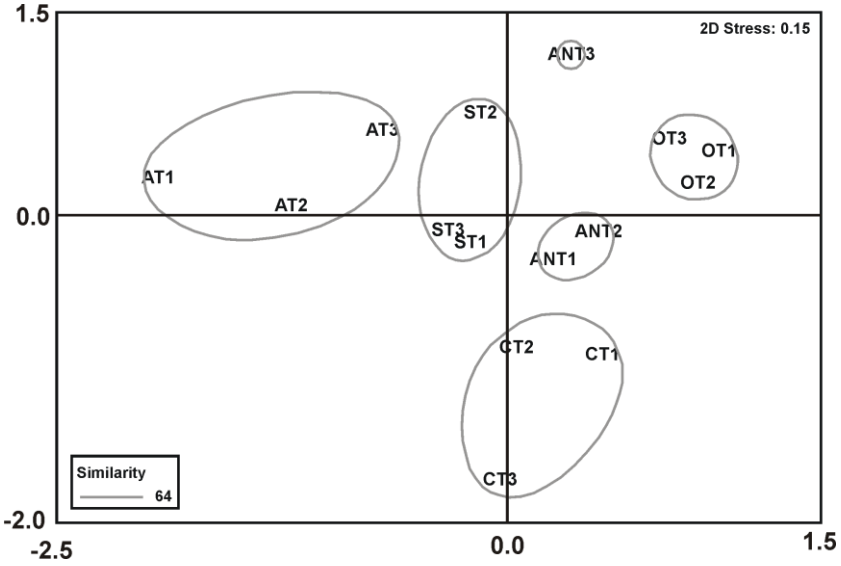

Figure 9 - Non-metric Multi-Dimensional Scaling (MDS) based on fungal communities assessed from DGGE OTUs on oak leaves decomposing in 5 streams of the Ave River basin (C, Couros; S, Selho; AN, Andorinhas; O, Oliveira and A, Agra). T1, T2 and T3 represent 9, 23 and 43 days of oak leaf immersion at each stream, respectively.

Results were supported by ANOSIM tests that indicated that community structure based on fungal OTUs significantly differ between streams (global R = 0.913). In addition, pairwise tests indicated that maximum differences in fungal communities were found between Oliveira *versus* Couros, Selho or Agra and between Agra and Couros (R = 1, for all comparisons). Furthermore, Selho also differed from Couros and Agra (R = 0.888 and 0.815, respectively) and Andorinhas differed from Agra and Couros (R = 0.888 and 0.852, respectively). To a less extent but still significant, fungal community structure also differed on leaves decomposing in Andorinhas *versus* Oliveira or Selho (R = 0.777).

3.7. Invertebrate diversity

The richness of invertebrate taxa on oak leaves varied between 1.9 in Agra Stream and 3.8 taxa in Selho stream (Table 9), and increased by the following order: Agra < Andorinhas < Oliveira < Couros < Selho.

Table 9 - Two-way ANOVAs on the effects of the stream, time and interaction between stream and time on richness of invertebrate taxa.

Treatment	SS	DF	MS	F	P
Stream	22.4	4	5.5889	6.3	0.0008
Time	0.9	2	0.4667	0.5	0.596
Stream x Time	22.8	8	2.8556	3.2	0.009
Error	26.7	30	0.889		

The stream and interaction between time and stream significantly affected invertebrate taxa on oak leaves (2-way ANOVA, Table 9).

The taxon richness was significantly higher in the highly eutrophic stream Selho than in Andorinhas, Oliveira and Agra streams (Tukey's post-tests, $P<0.05$). Overall taxon richness was higher in Selho and Andorinhas (9 taxa) and Pielou's was higher in Andorinhas and Agra streams (J' from 0.9 to 1.0, except for Andorinhas after 43 days of leaf immersion), while Shannon's diversity index was highest in Selho and Andorinhas stream (H' from 0.5 to 2.1, except for Andorinhas after 43 days of leaf immersion). On the contrary, the lowest values were found associated with oak leaves decomposing in the Couros stream (J' from 0.2 to 0.5; H' from 0.2 to 0.3). Furthermore, lower taxon richness was found in Couros and Agra streams (up to 4 taxa).

The contribution of invertebrate taxa also varied between streams (Table 10). Individuals from the family Chironomidae were dominant in the hypertrophic stream Couros, while Tubificidae were dominant in the highly eutrophic stream Selho. Trichoptera taxa were dominant in the moderately eutrophic stream Andorinhas, while in Oliveira stream the dominant families were Plecoptera and Ephemeroptera. At the oligotrophic stream Agra, dominant taxa belonged to the Sericostomatidae family.

Table 10 - Percentage of contribution of each invertebrate taxa to the total number of individuals on oak leaves immersed in 5 streams of the Ave River basin (Couros, Selho, Andorinhas, Oliveira and Agra). T1, T2 and T3 represent 9, 23 and 43 days of oak leaf immersion in each stream, respectively.

	Couros			Selho			Andorinhas			Oliveira			Agra		
Invertebrates taxon	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Diptera															
Chironomidae															
Chironomini	95.1	87.8	89.3	7.4	2.0										
Orthocladiinae								8.3							
Tanypodinae				2.1	0.6										
Dixidae															
Tipulidae												16.7	50.0		33.3
Athericidae													50.0		
Atherix															
Oligochaeta															
Tubificidae	4.9	11.8	4.6	44.2	87.2	36.7									
Lumbricidae						16.7									
Tabanidae (Larvae)			2.8												
Gastropoda															
Physidae				14.7											
Physa				20.0	7.8	23.3									
Lymnaeidae				5.3											
Galba truncata				1.1											
Hirudinea															
Hirudidae		0.4	3.3	4.2	2.4	3.3									
Glossiphoniidae				1.1											
Decapoda															
Cambaridae															
Procambarus clarkii						10.0	14.3	8.3							
Trichoptera															
Polycentropodidae							14.3								
Limnephilidae							42.9							37.5	
Ecnomidae								8.3							
Hydropsychidae						3.3									
Leptidostomatidae								8.3							
Sericostomatidae							14.3	25.0	100.0			33.3		62.5	50.0
Odonata															
Calopterygidae							14.3								
Libellicidae								8.3							
Plecoptera															
Nemouridae															
Nemoura								8.3		50.0	50.0				
Perlodidae															
Leuctridae												50.0			16.7
Ephemeroptera															
Leptophlebiidae								8.3		50.0	50.0				
Coleoptera															
Dysticidae						6.7		16.7							
Taxon richness	2.0	2.0	4.0	6.0	4.0	6.0	5.0	8.0	1.0	2.0	4.0	3.0	2.0	2.0	4.0
Pielou's evenness (*J'*)	0.4	0.5	0.2	0.9	0.4	0.8	0.9	1.0	-	1.0	0.9	1.0	1.0	1.0	0.9
Shannon index (*H'*)	0.3	0.3	0.2	1.6	0.5	1.4	1.5	2.1	0.0	0.7	1.2	1.1	0.7	0.7	1.3

The Non-metric MDS analyses based on invertebrate communities indicated that community structure differed between streams as suggested by the presence of 4 groups (overlay clusters) with 30% of similarity in the ordination plot (Figure 10): 1) communities from the oligotrophic stream Agra (9 days); 2) communities from the oligotrophic stream Agra (23 and 43 days), moderate eutrophic stream Oliveira (43 days) and moderately eutrophic stream Andorinhas (9 and 43 days); 3) communities from the moderately eutrophic stream Andorinhas (23 days) and moderate eutrophic stream Oliveira (9 and 23 days); 4) communities from highly eutrophic stream Selho and hypertrophic stream Couros.

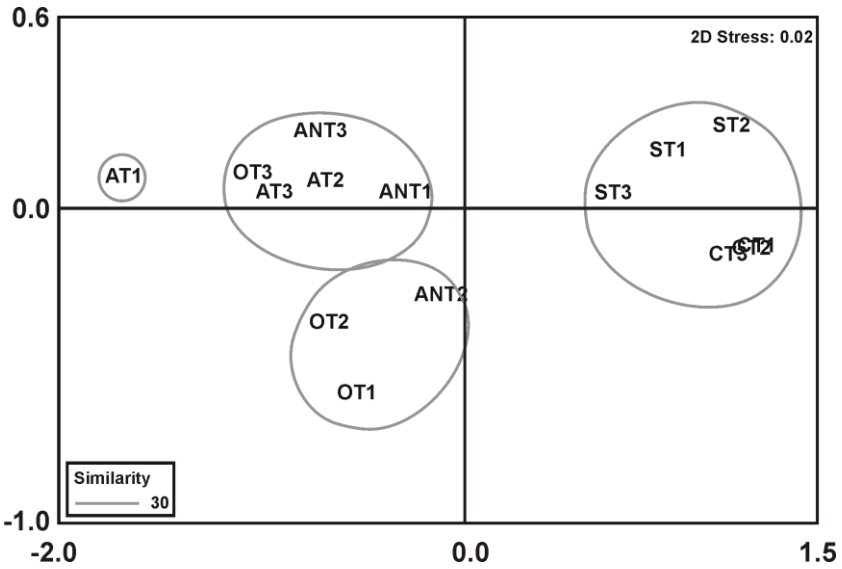

Figure 10 - Non-metric Multi-Dimensional Scaling (MDS) based on invertebrate communities on oak leaves decomposing at 5 streams of the Ave River basin. (C, Couros; S, Selho; AN, Andorinhas; O, Oliveira and A, Agra). T1, T2 and T3 represent 9, 23 and 43 days of oak leaf immersion at each stream, respectively

These results were supported by ANOSIM tests that indicated that community structure based on invertebrate taxa significantly differed between streams (global R = 0.636). In addition, pairwise tests indicated that maximum differences were attained between Andorinhas and Couros or Selho (R = 1, for both comparisons). Furthermore, Couros stream significantly differed from Selho (R = 0.889) and invertebrate communities in both streams differ from Oliveira or Agra (R = 0.833, for all comparisons). In contrast, Andorinhas stream did not significantly differ from Oliveira or Agra as suggested by the low values of R (R = 0.167 and 0.093, respectively). Moreover, no significant differences were also found between invertebrate communities decomposing at Oliveira and Agra (R = 0.037).

4. Discussion

In the current study, we assessed the effects of eutrophication on plant-litter decomposition in streams and its associated biota – fungi and macroinvertebrates. For accomplishing our objectives, oak leaves were immersed in 5 streams belonging to the Ave River basin and a PCA analysis, based on physical and chemical water parameters ordinated streams according to an eutrophication gradient as follows: 1) Agra, the most oligotrophic stream, 2) Oliveira and Andorinhas, moderately eutrophic streams, 3) Selho, a highly eutrophic stream and 4) Couros a hypertrophic stream.

Eutrophication significantly altered leaf decomposition rates and the community structure of associated biota. In general, lower values of leaf decomposition rates and fungal biomass were found in the most eutrophic streams (Selho and Couros), while the highest values were found in the moderately eutrophic streams (Oliveira and Andorinhas). The slight reduction in leaf decomposition rates observed in Andorinhas, in comparison with Oliveira, was probably due to high precipitation events that buried the bags at least for a period of one week in the last sampling date.

Fungal reproduction on decomposing leaves was lower in the hypertrophic stream (Couros) and in the oligotrophic stream (Agra), than in the other streams, suggesting that fungal reproduction appeared to respond to eutrophication according to a hump-shaped model as found by Geraldes (2011). Others authors (e.g., Duarte *el al.,* 2009; Pascoal *et al.,* 2005b) also found that fungal reproduction is quite sensitive to pollution and might be used as a tool to assess the effect of eutrophication in freshwater ecosystems. However, aquatic fungal communities can be sensitive to other environmental factors as well, such as temperature, substrate availability or a combination of both (e.g. Nikolcheva and Bärlocher, 2005). Furthermore, caution has to be taken because similar results for fungal reproduction were found in both hypertrophic and oligotrophic streams, which may bias the interpretation.

Although moderate eutrophication is reported to stimulate leaf decomposition rates and the activity of microorganisms associated with decomposing leaves (Ferreira *et al.,* 2006), the presence of highly toxic compounds in hypertrophic streams, such as high amounts of nitrites and ammonia, were previously reported to have an inhibitory effect on plant-litter decomposition and fungal biomass and sporulation (Baldy *et al.,* 2007; Duarte *et al.,* 2009). In fact, the levels of nitrites and ammonia were significantly higher in Couros than in the other streams (3.9 to 31 and

4.3 to 390 times higher, respectively). Moreover, the low oxygen concentration and the high amounts of sediments and mud found in the most hypertrophic stream (Couros) might have led to anoxic conditions, as suggested by the dark color of leaves, and this may reduce fungal activity (Duarte *et al.,* 2009; Pascoal and Cássio, 2004). In addition, in cases where nitrogen and phosphorus are not limiting the increase of their concentrations in the stream water may not stimulate litter decomposition or the activity of associated organisms (Grattan and Suberkroop, 2001; Ferreira *et al.,* 2006; Royer and Minshall, 2001).

In our study, eutrophication also altered the structure of fungal and invertebrate communities' on decomposing leaves. Fungal diversity assessed through the identification of conidia was severally reduced in the Couros stream (2 sporulating species *versus* 5, 7, 12 and 14 sporulating species found at Agra, Selho, Andorinhas and Oliveira, respectively). This is not totally surprising since it is recognized that changes in water chemistry can alter microbial community structure on plant-litter decomposing in freshwaters (Pascoal *et al.,* 2003; Pascoal and Cássio, 2004; Baldy *et al.,* 2007; Duarte *et al.,* 2009; Solé *et al.,* 2008; Artigas *et al.,* 2008). The concentrations of nitrites, phosphates and ammonia were 31, 42 and 390 times higher, respectively, in the most hypertrophic stream Couros than in the most oligotrophic stream Agra. In addition, in Selho, the second highest eutrophic stream, the levels of nitrates were 25 times higher than in Agra stream. Both Selho and Couros streams are very close to each other and have the influence of the city of Guimarães, which has high intensive agricultural activities. These differences in the trophic status are enough to explain the alterations in the structure of fungal communities'. Based on conidial identification, the relative contribution of each fungal species to the total conidium production varied along the gradient of eutrophication. In the hypertrophic stream (Couros), the dominant species was *Dimorphospora foliicola*, which was also abundant in the Selho stream. This species is reported to be abundant and even dominant in eutrophic streams in Northwest Portugal (Duarte *et al.,* 2008, 2009; Pascoal *et al.,* 2003, 2005b). Furthermore, Gulis and Suberkroop (2003) reported that the contribution of *D. foliicola* to the total conidial production increased in a nutrient-enriched stream reach. On the other hand, *Tetrachaetum elegans* and *Articulospora tetracladia* were dominant on oak leaves decomposing at the less eutrophic streams Andorinhas, Oliveira and Agra. *Articulospora tetracladia* is reported to have a worldwide distribution (Pascoal *et al.,* 2005b, c; Nikolcheva *et al.,* 2003; Gulis and Suberkroop, 2003) but it appears to be relatively sensitive to eutrophication (Pascoal *et al.,* 2005b; Duarte *et al.,* 2009). Our results also support this finding since spores from this species

were completely absent on leaves decomposing in Couros and Selho, the most eutrophic streams. Our results, as those found in previous studies conducted in streams of Northwest Portugal (Pascoal *et al.,* 2005b; Duarte *et al.,* 2008, 2009), suggest that some aquatic fungal species, such as *D. foliicola*, might become dominant on plant-litter decomposing in streams impacted by eutrophication. The increased contribution of certain species to total spore production in eutrophic streams is accompanied by a decline of the dominant species, such as *A. tetracladia* (current and previous studies) or *T. elegans* (current study).

The fungal community structure based on DGGE OTUs also indicated differences between streams. However, fungal diversity based on DGGE OTUs was different from that assessed using the conidial identification, the traditional technique; fungal diversity was highest in Oliveira and lowest in Agra; and Andorinhas showed lower diversity than the most hypertrophic streams (Couros and Selho). In spite of these differences, a higher diversity was generally found in all streams by DGGE (22 to 40 different OTUs); and these differences between traditional and molecular techniques were more prominent in eutrophic streams. As found in previous studies, the combination of both techniques proved to be useful to assess the impacts of eutrophication on fungal communities on plant-litter in streams (Duarte *et al.*, 2009). In particular, DGGE was valuable in assessing the diversity of fungi associated with plant-litter in the hypertrophic stream Couros, where sporulation was strongly inhibited.

Niyogi and collaborators (2002) proposed that under a gradient of stress biodiversity is usually more sensitive to stress, whereas biomass and function are stable or increase under low to moderate stress and decrease only under high stress levels (Niyogi *et al.*, 2002). Changes in function are predicted to follow changes in biomass across a gradient of stress, and suppression of function is related to suppression of biomass, rather than loss of biodiversity. In part, our results, at least for fungal communities followed this model. If we consider that the most oligotrophic stream Agra is our reference stream, an increase in leaf decomposition rates, fungal biomass, sporulation and diversity were found in Oliveira and Andorinhas, which have low to moderate levels of eutrophication. Although no differences were found in leaf decomposition rates in Couros comparing to Agra, fungal biomasses and diversity were lower in the former stream. If this high level of eutrophication is maintained or increases in Couros, we may predict that within few years leaf decomposition rates decreases.

Benthic macroinvertebrates have being widely used as bioindicators of eutrophication in streams (Pascoal *et al.,* 2001; 2003; Castela *et al.,* 2008; Baldy *et al.,* 2007). In the current

study, macroinvertebrate communities were also sensitive to eutrophication and able to discriminate the different streams. Individuals from Trichoptera and Plecoptera, reported as sensitive to organic pollution (Resh and Jackson, 1993), were mostly found in the oligotrophic stream Agra and moderately eutrophic stream Andorinhas, while tolerant taxa such as Diptera and Oligochaeta (Merrit and Cummins, 1996), were mostly found in the most eutrophic streams (Selho and Couros). In our study, the biomass of invertebrates increased in the highly eutrophic stream Selho and in the hypertrophic stream Couros. This agrees with previous studies, where a high density of invertebrates was found at the most polluted sites, due to the high abundances of chironomids and oligochaetes (Pascoal *et al.,* 2001, 2003, 2005a; Pereira, 2010; Robinson and Gessner, 2000). On the other hand, the average invertebrate richness was higher in the highly eutrophic stream Selho and decreased along the gradient of eutrophication. These unexpected results might be explained by the low number of invertebrates present on leaves in autumn-winter leading to possible biased analyzes. Shredder biomass was higher in moderate eutrophic streams (Andorinhas and Oliveira) and the oligotrophic stream Agra than in the most eutrophic streams (Selho and Couros). Shredders are reported to be sensitive to eutrophication (Pascoal *et al.,* 2001, 2003) and can be completely absent in hypertrophic streams (Baldy *et al.,* 2007; Pascoal *et al.,* 2005a). Our results also support this since no shredders were found on oak leaves decomposing in the hypertrophic stream Couros. Although chironomids and oligochaetes are reported to contribute to leaf breakdown (Graça, 2001; Pascoal *et al.*, 2003), their influence on leaf decomposition appears to be minor in Couros and Selho streams. In these streams high abundance of oligochaetes and chironomids were accompanied by reduced leaf decomposition rates. Oligochaetes and chironomids are known to feed on FPOM and use leaves as refugee and can contribute to leaf decomposition due to their movements (Chauvet *et al.,* 1993; Pascoal *et al.,* 2003).

Overall, in our study, eutrophication was responsible for differences in fungal biomass and sporulation and in invertebrate biomass on decomposing leaves in the five streams of the Ave River basin. Leaf decomposition rates, fungal biomass and reproduction were higher in the moderate eutrophic streams than in the most oligotrophic and hypertrophic streams, while higher biomasses of invertebrates were found in the most polluted streams. Benthic invertebrate communities were sensitive to water quality and were able to discriminate the streams according to eutrophication level. In the most eutrophic streams, shredders were completely or almost absent. This together with a reduction in fungal activity can explain the low decomposition rates

found in these streams. In addition, fungal community structure, assessed as conidial counts or DGGE OTUs, was also affected by changes in the concentration of inorganic nutrients in the stream water. DGGE was valuable in assessing fungal diversity in the most eutrophic stream, where sporulation was strongly inhibited.

In a near future, moving to the next generation sequencing techniques, such as pyrosequencing, might help to reduce the biased interpretations regarding fungal identification enforcing the use of fungal communities as a tool to assess the integrity of freshwaters. Combining structural measures based on aquatic fungal and benthic invertebrate communities with functional measures, such as leaf-litter decomposition, can enhance our ability to assess the impacts of eutrophication in freshwater ecosystems.

References

Abelho, M. (2001) From Litterfall to Breakdown in Streams: A Review. *The Scientific World*, 1: 656-680.

Allan, J.D., Castillo, M.M. (2007) Stream ecology: structure and function of running waters. 2nd ed., Springer, Dordrecht, The Netherlands, 436 pp.

Alves, A., Boaventura, R., Soares, H. (2009) Evaluation of heavy metals pollution loadings in the sediments of the Ave river basin (Portugal). *Soil and Sediment Contamination,* 18: 603–618.

Amend, A.S., Seifert, K.A., Bruns, T.D. (2010) Quantifying microbial communities with 454 pyrosequencing: does read abundance count? *Molecular Ecology,* 19: 5555-5565.

Araújo, M.F., Valério, P., Jouanneau, J-M. (1998) Heavy metal assessment in sediments of the Ave River Basin (Portugal) by energy-dispersive X-Ray Fluorescence Spectrometry. *X-Ray Spectrometry,* 2: 305-312.

Artigas, J., Romaní, A.M., Sabater, S. (2008) Effect of nutrients on the sporulation and diversity of aquatic hyphomycetes on submerged substrata in a Mediterranean stream. *Aquatic Botany,* 88: 32–38.

Baldy, V., Gobert, V., Guérold, F., Chauvet, E., Lambrigot, D., Charcosset, J-Y. (2007) Leaf litter breakdown budgets in streams of various trophic status: effects of dissolved inorganic nutrients on microorganisms and invertebrates. *Freshwater Biology*, 52:1322–35.

Barbour, M.T., Gerritsen, J., Snyder, B.D., Stribling, J.B. (1999) *Rapid bioassessment protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates and fish*. 2nd ed. EPA 841-B-99-002. U.S. Environmental Protection Agency, Office of Water, Washington, D.C., USA.

Bärlocher, F. (2005) Freshwater fungal communities. In: Deighton J., Oudemans P., White J. (Eds.) *The fungal community: its organization and role in the ecosystem*, 3rd ed., Taylor and Francis, CRC Press, Boca Raton, Florida, pp. 39-59.

Bärlocher, F. (2007) Molecular approaches applied to aquatic hyphomycetes. *Fungal Biology Reviews*, 21: 19-24.

Bärlocher, F. (2010) Molecular approaches promise a deeper and broader understanding of the evolutionary ecology of aquatic hyphomycetes. *Journal of the North American Benthological Society,* 29: 1027-1041.

Bärlocher, F., Kendrick, B. (1975) Leaf Conditioning by Microorganisms. *Oecologia,* 20: 359-362.

Bärlocher, F., Porter, C.W. (1986) Digestive enzymes and feeding strategies of three invertebrates. *Journal of the North American Benthological Society,* 5: 58–66.

Benfield, E.F. (1996) Leaf breakdown in stream ecosystems. In Hauer, F.R., Lamberti, G.A. (Eds.) *Methods in stream ecology*, Academic Press, San Diego, California, USA, pp 579–589.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R.H., Uroz, S., Martin, F. (2009) 454 pyrosequencing analyses of forest soils reveal and unexpected high fungal diversity. *New Phytologist,* 184: 449-456.

Cairns, J.Jr. (1975) Quantification of biological integrity. In: Ballentine, R. K., Guarraie, L.J. (Eds.) The integrity of water: a symposium. U.S. Environmental Protection Agency, Washington, D.C. (No report number). pp.171-185

Casas, J., Gessner, M.O. (1999) Leaf litter breakdown in a Mediterranean stream characterized by travertine precipitation. *Freshwater Biology,* 41: 781–793.

Castela, J., Ferreira, V., Graça M.A.S. (2008) Evaluation of stream ecological integrity using litter decomposition and benthic invertebrates*. Environmental Pollution,* 153: 440–449.

Chauvet, E., Giani, N., Gessner, M.O. (1993) Breakdown and invertebrate colonization of leaf-litter in two contrasting streams significance of oligochaetes in a large river. *Canadian Journal of Fisheries and Aquatic Sciences*, 50: 488-495.

Chergui, H., Pattee, E. (1991) An experimental study of the breakdown of submerged leaves by hyphomycetes and invertebrates in Morocco. *Freshwater Biology,* 26: 97–110.

Clarke, K.R., Gorley, R.N. (2006) PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

Cuffney, T.F., Wallace, J.B., Lugthart, G.J. (1990) Experimental evidence quantifying the role of benthic invertebrates in organic matter dynamics of headwater stream. *Freshwater Biology*, 23: 281-300.

Cummins, K.W. (1973) Trophic relations of aquatic insects. *Annual Review of Entomology,* 18: 183–206.

Cummins, K.W. (1974) Structure and function of stream ecosystems. *BioScience*, 24:631–641.

Dangles, O., Chauvet, E. (2003) Effects of stream acidification on fungal biomass in decaying beech leaves and leaf palatability. *Water Research,* 37: 533-538.

Das, M., Royer, T.V., Leff, L.G. (2008) Fungal communities on decaying leaves in streams: a comparison of two leaf specie. *Mycological Progress,* 7: 267-275.

Del Arco, A.I., Ferreira, V., Graça, M.A.S. (2012) The performance of biological indicators in assessing the ecological state of streams with varying catchment urbanisation levels in Coimbra, Portugal. *Limnetica,* 31: 141-154.

DeLong, E.F. (2009) The microbial ocean from genomes to biomes. *Nature*, 459: 200-206.

Dieterich, M., Anderson, N.H., Andersson, T.M. (1997) Shredder-collector interactions in temporary streams of western Oregon. *Freshwater Biology,* 38: 387–393.

Duarte, S., Pascoal, C., Alves, A., Correia, A., Cássio, F. (2010) Assessing the dynamic of microbial communities during leaf decomposition in a low-order stream by microscopic and molecular techniques. *Microbiological Research,* 165: 351-362.

Duarte, S., Pascoal, C., Garabétian, F., Cássio F., Charcosset, J-Y. (2009) Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. *Applied and Environmental Microbiology* , 75: 6211-6221.

Duarte, S., Seena, S., Bärlocher, F., Cássio, F., Pascoal, C. (2012) Preliminary insights into the phylogeography of six aquatic hyphomycete species. Public Library of Science One 7 (9), e45289.

Duarte, S., Seena, S., Bärlocher, F., Pascoal, C., Cássio, F. (2013) A decade´s perspective on the impact of DNA sequencing on aquatic hyphomycete research. *Fungal Biology Reviews,* 27: 19-24.

Duarte, S., Pascoal, C., Cássio, F. (2008). High diversity of fungi may mitigate the impact of pollution on plant litter decomposition in streams. *Microbial Ecology*, 56: 688-695.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A-H., Soto, D., Stiassny, M.L.J., Sullivan, C.A. (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews,* 81: 163–182.

Edwards, R.A., Rodriguez-Brito, B., Wegley, L., Haynes, M., Breitbart, M., Peterson, D.M., Saar, M.O., Alexander, S., Alexander Jr, E.C., Rohwer, F. (2006) Using pyrosequencing to shed light on deep mine microbial ecology. *BMC Genomic,* 7: 57, 13 pp.

Elosegi, A. (2005) Leaf retention. In: Graça, M.A.S., Bärlocher, F., Gessner, M.O. (Eds.). Methods to study litter decomposition: a practical guide. Springer, Dordrecht, The Netherlands, pp. 13-18.

Ferreira, V., Gulis, V., Graça, M.A.S. (2006) Whole-stream nitrate addition affects litter decomposition and associated fungi but not invertebrates. *Oecologia,* 149: 718–729.

Fisher, S.G., Likens, G.E. (1973) Energy flow in Bear Brook, New Hampshire: anintegrative approach to stream ecosystem metabolism. *Ecological Monographs,* 43: 421–439.

Geraldes, P. (2011) *Fungal communities and functional measures as indicators of stream ecosystem health*. Master Thesis in Ecology. University of Minho.

Gessner, M.O., Chauvet, E. (1993) Ergosterol-to-biomass conversion factor for aquatic hyphomycetes. *Applied and Environmental Microbiology*, 59: 502-507.

Gessner, M.O., Chauvet, E. (2002) A case for using litter breakdown to assess functional stream integrity. *Ecological Applications*, 12: 498-510.

Gessner, M.O., Chauvet, E., Dobson, M. (1999) A perspective on leaf litter breakdown in streams. *Oikos,* 85: 377–384.

Golladay, S.W., Webster, J.R., Benfield, E.F. (1983) Factors affecting food utilization by a leaf shredding aquatic insect: leaf species and conditioning time. *Holartic Ecology,* 6: 157-162.

Graça, M.A.S. (1993) Patterns and processes in detritus-based stream systems. *Limnologica,* 23:107 pp.

Graça, M.A.S. (2001) The role of invertebrates on leaf litter decomposition in streams - A review. *International Review of Hydrobiology*, 86:383-393.

Graça, M.A.S., Maltby, L., Calow, P. (1993) Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus*. I. Feeding strategies. *Oecologia*, 93: 139-144.

Grattan, R.M., Suberkropp, K. (2001) Effects of nutrient enrichment on yellow poplar leaf decomposition and fungal activity in streams. *Journal of the North American Benthological Society,* 20: 33-43.

Gulis, V., Suberkropp, K. (2003) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshwater Biology,* 48: 123-134.

Gulis, V., Suberkropp, K. (2004) Effects of whole-stream nutrient enrichment on the concentration and abundance of aquatic hyphomycete conidia in transport. *Mycologia*, 96: 57-65.

Ingold, C.T. (1942) Aquatic hyphomycetes of decaying alder leaves. *Transactions of the British Mycological Society,* 25: 339-417.

Iwen, P.C., Hinrichs, S.H., Rupp, M.E. (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Medical Mycology*, 40: 87-109.

Karr, J.R. (1991) Biological integrity: a long-neglected aspect of water resource management. *Ecological Applications,* 1: 66-84.

Karr, J.R., Dudley, D.R. (1981) Ecological perspective on water-quality goals. *Environmental Management,* 5: 55-68.

Kiran, U., (1996) Fungi and food preferences of aquatic invertebrates. *National Academy Scienc Letters,* 19: 188-190.

Klug, M.J., Kotarski, S. (1980) Bacteria associated with the gut tract of larval stages of the aquatic cranefly tipula abdominalis (Diptera; Tipulidae). *Applied and Environmental Microbiology,* 40: 408-416.

Kruskal, J.B., Wish, M. (1978) Multidimensional Scaling. Sage Publications, Inc. pp. 69

Lecerf, A., Usseglio-Polatera, P., Charcosset, J.Y., Bracht, B., Chauvet, E. (2006) Assessment of functional integrity of eutrophic streams using litter breakdown and benthic invertebrates. *Archiv. Hydrobiologie,* 165: 105-126.

Lecerf, A., Dobson, M., Dang, C.K., Chauvet, E. (2005) Riparian plant species loss alters trophic dynamics in detritus-based stream ecosystems. *Oecologia,* 146:432-442.

Legendre, P., Legendre, L. (1998) Numerical Ecology. Developments in environmental modeling, 20, 2nd ed. Elsevier, Amsterdam, The Netherlands, 870 pp.

Merrit, R.W., Cummins, K.W. (1996) An introduction to aquatic insects of North America, 3rd ed. Kendall/ Hunt Publishing Company, Iowa, USA.

Mesquita, A., Pascoal, C., Cássio, F. (2007) Assessing effects of eutrophication in streams based on breakdown of eucapypt leaves. *Fundamental and Applied Limnology,* 168: 221-230.

Metcalfe-Smith, J. L. (2009) Biological water quality assessment of rivers: use of macroinvertebrate communities. In: *The Rivers Handbook: Hydrological and Ecological Principles*, Volume Two (eds P. Calow and G. E. Petts), Blackwell Science Ltd, Oxford, UK.

Mille-Lindblom, C., Tranvik, L.J. (2003) Antagonism between bacteria and fungi on decomposing aquatic plant litter. *Microbial Ecology*, 45: 173-182.

Moreirinha, C., Duarte, S., Pascoal, C., Cássio, F. (2011) Effects of cadmium and phenanthrene mixtures on aquatic fungi and microbially mediated leaf litter decomposition. *Archives of Environmental Contamination and Toxicology*, 61: 211-219.

Mulholland, P.J., Fellows, C.S., Tank, J.L., Grimm, N.B., Webster J.R., Hamilton, S.K., Martí, E., Ashkenas, L., Bowden W.B., Dodds, W.K., McDowell, W.H., Paul, M.J., Peterson, B.J. (2001) Inter-biome comparison of factors controlling stream metabolism. *Freshwater Biology,* 46: 1503-1517.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gelelectrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Applied and Environmental Microbiology*, 59: 695–700.

Muyzer, G., Smalla, K. (1998) Application of Denaturating Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwnhoek*, 73: 127-141.

Myers, R.M., Fischer, S.G., Lerman, L.S., Maniatis, T. (1985) Nearly all single base substitutions in DNA fragments joined to a GC clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research,* 13: 3131–3145.

Myers, R.M., Maniatis, T., Lerman, L.S. (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods of Enzymology,* 155: 501–527.

Nikolcheva, L.G., Bärlocher, F. (2005) Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional versus molecular evidence. *Environmental Microbiology,* 7: 270-280.

Nikolcheva, L.G., Bourque, T., Bärlocher, F. (2005) Fungal diversity during initial stages of leaf decomposition in a stream. *Mycological Research,* 109: 246-253.

Nikolcheva, L.G., Cockshutt, A.M., Bärlocher, F. (2003) Determining diversity of freshwater fungi on decomposing leaves: comparison of traditional and molecular approaches. *Applied and Environmental Microbiology*, 69: 2548-2554.

Nilsson, R.H., Ryberg, M., Abarenkov, K., Sjökvist, E., Kristiansson, E. (2009) The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiology Letters,* 296: 97-101.

Niyogi, D.K., Lewis, Jr, W.M., Mcknight, D.M. (2002) Effects of stress from mine drainage on diversity, biomass, and function of primary producers in mountain streams. *Ecosystems,* 5: 554-567.

Pascoal, C., Cássio, F. (2004) Contribution of fungi and bacteria to leaf litter decomposition in a polluted river. *Applied and Environmental Microbiology,* 70: 5266-5273.

Pascoal, C., Cássio, F., Gomes, P. (2001) Leaf breakdown rates: a measure of water quality?. *International Review of Hydrobiology,* 86: 407-416.

Pascoal, C., Cássio, F., Marcotegui, A., Sanz, B., Gomes, P. (2005a) Role of fungi, bacteria, and invertebrates in leaf litter breakdown in a polluted river. *Journal of the North American Benthological Society*, 24: 784-97.

Pascoal, C., Cássio, F., Marvanová, L. (2005b) Anthropogenic stress may affect aquatic hyphomycete diversity more than leaf decomposition in a low order stream. *Archiv für Hydrobiologie,* 162: 481-496.

Pascoal, C., Marvanová, L., Cássio F. (2005c). Aquatic hyphomycete diversity in streams of Northwest Portugal. *Fungal Diversity,* 19: 109-128.

Pascoal, C., Cássio, F., Nikolcheva, L., Bärlocher, F. (2010) Realized fungal diversity increases functional stability of leaf litter decomposition under zinc stress. *Microbiol Ecology,* 59: 84-93.

Pascoal, C., Pinho, M., Cássio, F., Gomes, P. (2003) Assessing structural and functional ecosystem condition using leaf breakdown: studies on a polluted river. *Freshwater Biology*, 48: 2033–2044.

Pereira, A. (2011) *Effects of plant-litter quality on litter decomposition by benthic invertebrates along a gradient of eutrophication*. Master Thesis in Ecology. University of Minho.

Petersen, R.C., Cummins, K.W. (1974) Leaf processing in a woodland stream. *Freshwater Biology,* 343-368.

Pradhan, A., Seena, S., Pascoal, C., Cássio, F. (2011). Can metal nanoparticles be a threat to microbial decomposers of plant litter in streams? *Microbial Ecology*, 62: 58-68.

Resh, V.H., Jackson, J.K. (1993) Rapid bioassessment approaches to biomonitoring using benthic invertebrates. In: Rosenberg, D.M., Resh, V.H. (Eds.) *Freshwater Biomonitoring and Benthic Macroinvertebrates*. Chapman & Hall, Thompson Publishing, New York, 195–223.

Robinson, C.T., Gessner, M.O. (2000) Nutrient addition accelerates leaf breakdown in an alpine springbrook. *Oecologia*, 122: 258-263.

Rosenberg, D.M., Resh, V.H. (Eds.) (1993) Freshwater biomonitoring and benthic macroinvertebrates. Chapman and Hall, New York.

Royer , T.V., Minshall, G.W. (2001) Effects of nutrient enrichment and leaf quality on the breakdown of leaves in a hardwater stream. *Freshwater Biology*, 46: 603-610.

Royer, T.V., Minshall, G.W. (2001) Effects of nutrient enrichment and leaf quality on the breakdown of leaves in a hardwater stream. *Freshwater Biology,* 46: 603-610.

Sala, O.E., Chapin, F.S., Armesto, J.J., Berlow, R., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, I.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D., Mooney, H.A., Oesterheld, M., Poff, N.L., Sykes, M. T., Walker, B.H., Walker, M. Wall, D.H. (2000) Global biodiversity scenarios for the year 2100. *Science,* 287: 1770–1774.

Schadt, C.W., Martin, A.P., Lipson, D.A., Schmidt, S.K. (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science,* 301: 1359-1361.

Schoch, C.L, Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., and Fungal Barcoding Consortium. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences USA,* 109: 6241-6246.

Seena, S., Wynberg, N., Bärlocher, F. (2008) Fungal diversity during leaf decomposition in a stream assessed through clone libraries. *Fungal Diversity,* 30: 1-14.

Soares, H., Boaventura, R., Machado, A., Esteves da Silva, J. (1999) Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): multivariate analysis of data. *Environmental Pollution,* 105: 311-323

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplored ''Rare biosphere''. *Proceedings of the National Academy of Sciences USA*, 103: 12115-12120.

Solé, M., Fetzer, I., Wennrich, R., Sridhar, K.R., Harms, H., Krauss, G. (2008) Aquatic hyphomycete communities as potential bioindicators for assessing anthropogenic stress. *Science of the Total Environment,* 389: 557-565.

Sridhar, K.R., Bärlocher, F. (2000) Initial colonization, nutrient supply, and fungal activity on leaves decaying in streams. *Applied and Environmental Microbiology,* 66: 1114-1119.

Sridhar, K.R., Duarte, S., Cássio, F., Pascoal, C. (2009) The role of early fungal colonizers in leaf-litter decomposition in Portuguese streams impacted by agricultural runoff. *International Review Hydrobiology*, 94: 399-409.

Suberkropp, K. (1998) Microorganisms and organic matter decomposition. – In: Naiman, R.J., Bilby, R.E. (eds) *River ecology and management: lessons from the Pacific coastal ecoregion*. – Springer, New York, pp. 120-143.

Suberkropp, K., Chauvet, E. (1995) Regulation of leaf breakdown by fungi in streams: influences of water chemistry. *Ecology*, 76: 1433-1445.

Suberkropp, K., Godshalk, G.L., Klug, M.J. (1976) Changes in thechemical composition of leaves during processing in a woodland stream. *Ecology,* 57: 720–7.

Tachet, H., Richoux, P., Bournaud, M., Usseglio-Polatera, P. (2010) Invertébrés d'eau douce: systématique, biologie, écologie. 1st ed. CNRS Editions, Paris, France.

Vandenkoornhuyse, P., Bauldauf, S.L., Leyval, C., Straczek, J., Young, J.P.W. (2002). Extensive fungal diversity in plant roots. *Science,* 295: 2051.

Vannote, R.L., Minshall, G.W., Cummins, K.W., Sedell, J.R., Cushing, C.E. (1980) The river continuum concept. *Canadian Journal of Fisheries and Aquatic Sciences,* 37: 130-137.

Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M. (1997) Human domination of Earth's ecosystems. *Science,* 277: 494-499.

Webster, J.B., Benfield, E.F. (1986) Vascular plant breakdown in freshwater eco-systems. *Annual Review of Ecology and Systematics,* 17: 567–594.

White, T.J., Bruns, S., Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR protocols; a guide to methods and applications, Academic Press Inc, New York, 345-322.

Wright, J.G., Sutcliffe, D.W., Furse, M.T. (2000) Assessing the biological quality of freshwaters. RIVPACS and other techniques. *The Freshwater Biological Association*, Cumbria, UK, pp. 400

Zar, J.H. (2009) Biostatistical analysis. 5th ed., Prentice-Hall, Englewood Cliffs, USA, pp. 944.

Zimmer, M., Topp, W. (1998) Microorganisms and cellulose digestion in the gut of the woodlouse *Porcellio scaber*. *Journal Chemical Ecology,* 24: 1397–1408.