FACTORS AFFECTING DECOMPOSITION OF SUBMERGED LITTER AND ASSOCIATED MICROBES AND INVERTEBRATES

BY

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We must treat each and every swamp, river basin, river and tributary, forest and field with the greatest care, for all these things are the elements of a very complex system that serves to preserve water reservoirs – and that represents the river of life.

Mikhail Gorbachev

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À minha Família,
e a Ti cuja existência iluminou
uma década da minha vida

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GENERAL INTRODUCTION

Amazingly, only *ca*. 0.007 % of the water covering our blue planet is readily accessible for direct human uses, as is the surface freshwater and shallow groundwater (UN, 1997). However, due to the increasing human needs, resulting from a growing population, the water in these freshwater reservoirs is threatened in both quantity and quality. Water withdrawals, mainly for agriculture, result in decreased stream/river discharges and groundwater depletion (UN, 1997, 2006). On the other hand, the intensive agriculture, high industrial development and low sanitation are factors that impair water quality all over the world (UN, 1997, 2006). In face of the current global warming (PCC, 2001) and increase in human population (UN, 2002) water of high quality is expected to become scarce (UN, 2006). To face this issue in Europe the European Community developed the Water Framework Directive, which induces the member countries to take all necessary measures to guarantee that all water bodies have high quality by the year 2015 (EU DIRECTIVE 2000/60/EC). The first step to accomplish this is the assessment of the ecological integrity of aquatic ecosystems.

Up to date, stream assessment protocols use ecological integrity of aquatic ecosystems as synonym of structural integrity, which they actually measure. Structural parameters of the benthic macroinvertebrate and diatom communities have been extensively used with this goal (METCALFE, 1989). Nevertheless, ecological integrity is composed of two fractions, structural and functional integrity (GESSNER & CHAUVET, 2002). Structure and function, which are interdependent, describe different aspects of the same system and should therefore be considered when evaluating ecosystem ecological integrity (GESSNER & CHAUVET, 2002). A good candidate to be used as a measure of functional integrity of aquatic ecosystems is the decomposition rate of submerged organic matter (PASCOAL *et al*., 2001, 2003; GESSNER & CHAUVET, 2002; DANGLES *et al*., 2004), which is a vital ecological process in low order, forested streams (VANNOTE *et al*., 1980; WEBSTER & MEYER, 1997).

In small streams running through forests primary production is limited due to the low water temperature and low solar irradiation of the water surface as a result of high riparian cover (MULHOLLAND *et al*., 2001). In these streams the primarily source of energy and carbon is allochthonous organic matter, i.e. leaves and other organic material produced by riparian trees (MOLINERO & POZO, 2004), and they are therefore highly heterotrophic (VANNOTE *et al*., 1980; MULHOLLAND *et al*., 2001). The decomposition of this organic matter is carried out by

an array of organisms including bacteria, aquatic fungi and shredding invertebrates (STEWART, 1992; BALDY *et al*., 1995; ROBINSON *et al*. 1998; HIEBER & GESSNER, 2002; GULIS & SUBERKROPP, 2003a, b). The rate at which organic matter is decomposed is affected by the interplay of both biotic and abiotic factors. Biotic factors include litter quality (ESCUDERO *et al*., 1991; BALDY *et al*., 1995; CHADWICK & HURYN, 2003) and presence and abundance of shredding invertebrates (CUFFNEY *et al.*, 1990; STEWART, 1992), while some abiotic factors are current velocity (CHERGUI & PATTEE, 1988; CANTON & MARTINSON, 1990; RADER *et al.,* 1994), water temperature (CHERGUI & PATTEE, 1990), dissolved oxygen (RAVIRAJA *et al*., 1998), dissolved nutrients (SUBERKROPP & CHAUVET, 1995; ROBINSON & GESSNER, 2000; GULIS & SUBERKROPP, 2003b), pH (DANGLES *et al*., 2004), toxic compounds (NIYOGI *et al*. 2001). Decomposition rates are in this way an integrative measurement of environmental conditions, including anthropogenic stress. However, if decomposition rates are to be used as an assessment tool of stream functional integrity one must discriminate between the effect of anthropogenic stress and natural variability. Also, one must identify the mechanisms by which environmental factors control litter decomposition.

The general objectives of the research described in this thesis were a) to identify the mechanisms ruling litter decomposition in streams and b) to assess the potential for decomposition rates of submerged litter to be used as an assessment tool to evaluate the functional integrity of streams (GESSNER & CHAUVET, 2002).

In **CHAPTER I** it was assessed the relative importance of physical fragmentation (due to current) and invertebrate fragmentation in leaf litter decomposition experiments using the coarse-fine mesh bag approach, in artificial channels where current velocity and invertebrate densities were manipulated. It was also assessed the effect of depth and current velocity, in summer and autumn, in leaf litter decomposition in a 4th order stream. The specific objective of this study was to evaluate the effect of inter habitat variability in the decomposition of submerged leaf litter and the usefulness of the coarse-fine mesh bag approach in evaluating the role of microbes *vs*. the role of invertebrates in litter decomposition.

In **CHAPTER II** it was assessed the effect of anthropogenic nutrient enrichment of stream water in the decomposition rates of submerged leaves, and associated fungal and invertebrate activity, and in traditional structural parameters based on the benthic macroinvertebrate community, using a reference-impacted stream pair design. The objective of this study was to evaluate the relationship between decomposition rates and dissolved

nutrients and the potential for leaf litter decomposition rates to be used as a tool to assess nutrient enrichment in streams.

CHAPTER III deals with a manipulative experiment consisting of an experimental nitrate enrichment of stream water to assess the relationship between nitrate and decomposition of three substrate types differing in initial nutrient quality, and on associated fungal and invertebrate parameters. Decomposition of submerged litter, and associated microbial and invertebrate activity, was followed along a nitrate gradient where concentrations were in the range observed in cultural enriched streams. In this study it was addressed the quantitative aspects of the response of decomposition and associated parameters to nutrient enrichment, such as the shape of the response curve or the existence of threshold or saturation phenomena within the nitrate concentration range.

In **CHAPTER IV** it was assessed the effect of the substitution of native deciduous forests by eucalyptus plantations on the decomposition of native leaf species and associated fungal communities, in Portugal and in Spain. The objective of this study was to evaluate the sensitivity of decomposition rates and fungal activity and diversity to changes in the composition of the riparian vegetation.

Except for water temperature, current velocity and suspended sediments, which can lead to mass loss by directly enhancing leaching and physical fragmentation of litter (CHERGUI & PATTEE, 1988, 1990), most other abiotic factors rule litter decomposition trough their effect on microbes (NIYOGI *et al*. 2001; GULIS & SUBERKROPP, 2003b; DANGLES *et al*., 2004). This leads to the need of understanding the effect of environmental factors on aquatic communities.

In **CHAPTER V** it was assessed the effect of current velocity and shredder presence in the structure and activity of the fungal communities colonizing alder leaves incubated in coarse and fine mesh bags in artificial channels. The objective of this study was to address the existence of a top-down (shredders) or a bottom-up (current) effect ruling the fungal communities associated with decomposing litter.

In **CHAPTER VI** it was assessed the effect of two contrasting nitrogen concentrations in water on the activity of the fungal communities colonizing balsa squares in microcosms. The objective of this study was to investigate if the activity of fungal communities associated with decomposing wood is stimulated with an increase in dissolved nutrients the same way as the ones associated with leaves.

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CHAPTER I

LEAF DECOMPOSITION IS AFFECTED BY INVERTEBRATE ACTIVITY BUT NOT BY PHYSICAL FRAGMENTATION

FERREIRA V., GRAÇA M.A.S., DE LIMA J.L.M.P. & GOMES R. 2006. Role of physical fragmentation and invertebrate activity in the breakdown rate of leaves. *Arch Hydrobiol* 165: 493–513

Photos:

Artificial channels, Dept. of Civil Engineering, University of Coimbra, 3030-219 Coimbra, Portugal

S. João stream, Lousã, Lousã Mountain, Portugal

Alder (*Alnus glutinosa* (L.) GAERTNER) leaves decomposing in coarse mesh bags

Sericostoma sp. (Trichoptera: Sericostomatidae) in artificial channels

LEAF DECOMPOSITION IS AFFECTED BY INVERTEBRATE ACTIVITY BUT NOT BY PHYSICAL FRAGMENTATION

ABSTRACT

In this study the relative importance of current velocity and invertebrate activities in the decomposition rate of alder (*Alnus glutinosa* (L.) GAERTNER) leaves was evaluated. Decomposition experiments were carried out in artificial channels, where current velocity and shredder presence were manipulated, and in a 4th order stream, in both summer and autumn, where litter bags were incubated in several sites differing in both depth and current velocity. Alder leaves incubated in artificial channels decomposed significantly faster in the presence of shredders than in their absence (k=0.0368 d^{-1} *vs.* k=0.0210 d^{-1} in low current and k= 0.0472 d^{-1} *vs.* k= 0.0219 d^{-1} in high current). However, current (up to 2.35 m s^{-1}) had no significant effect on decomposition rates. In channels without invertebrates, no significant differences in k values were found between coarse and fine mesh bags in high (0.20 m s^{-1}) and low (0.05 m s^{-1}) current. Leaves incubated in the stream during summer, in sites with current velocity ranging from 0.003 to 1.185 m s^{-1}, did not differ in their decomposition rates (k=0.0489 d^{-1} to k=0.0645 d^{-1}). In autumn, leaves exposed to high current (1.228 m s^{-1}) had faster decomposition rate (k=0.0417 d^{-1} *vs.* k=0.0136 d^{-1}), which may be related to sediment transport during this time of the year or to the tendency for higher number of shredders in high current-shallow sites.

Key-words: alder, artificial channels, current velocity, depth, shredders, stream

INTRODUCTION

In low order streams running through forests, the major energy source is allochthonous organic matter, i.e. leaves and other organic material produced by trees in the riparian zone (VANNOTE *et al.*, 1980; ABELHO, 2001). In the water, litter decomposition starts immediately and usually proceeds in three overlapping phases: (1) leaching of soluble compounds, which can lead to the loss of up to 42 % of the initial leaf mass (reviewed by ABELHO, 2001); (2) microbial decomposition, which can be responsible for the loss of up to 27 % of leaf mass (HIEBER & GESSNER, 2002); and (3) biotic and physical fragmentation. Biotic fragmentation results from the feeding activities of invertebrates, mainly shredders, which can result in up to 64 % of mass loss (GRAÇA, 2001; HIEBER & GESSNER, 2002).

Many studies on litter processing address the effect of microbes *vs.* invertebrates on litter decomposition, using fine and coarse mesh bags implanted in streams. These studies assume the differences in mass loss between bag types as a measure of invertebrate feeding since fine mesh bags exclude invertebrates. However, differences between bag types may also be due to physical fragmentation resulting from abrasion caused by current and transported sediments. This question can be addressed by isolating the effect of current velocity, using artificial channels where current velocity is controlled. This approach was used by CHERGUI & PATTEE (1988), CANTON & MARTINSON (1990), RADER *et al.* (1994) and VINGADA (1995), who reported high decomposition rates in high current. The effect of invertebrate activity on leaf litter decomposition was also addressed by CUFFNEY *et al.* (1990), STEWART (1992), JONSSON *et al.* (2001) and HURYN *et al.* (2002), by comparing decay rates of leaf species in streams differing in invertebrate densities. However, the compared streams could also differ in other factors besides invertebrate densities.

The relative importance of physical fragmentation and invertebrate feeding activities in experiments with coarse mesh bags is therefore still unclear. Recently, litter decomposition has been proposed to be used as an assessment tool to assess the functional health of aquatic ecosystems (PASCOAL *et al.*, 2001, 2003; GESSNER & CHAUVET, 2002; DANGLES *et al.*, 2004), and since this is usually done by using the litter bag approach it seems crucial to assess the sensitivity of this technique to physical fragmentation so that it would be possible to know to what extent decomposition rates reflect functional health and not physical conditions.

The objectives of this study were: (1) to investigate the relative importance of physical fragmentation *vs.* invertebrate fragmentation in leaf litter decomposition experiments using the coarse-fine mesh bag approach, in artificial channels where current velocity and invertebrate

densities were manipulated; (2) to assess the effect of increasing current velocities in leaf litter decomposition experiments, in artificial channels and (3) to evaluate the inter-habitat and temporal variability (depth and current velocity in summer and autumn) in leaf litter decomposition in a 4th order stream.

METHODS

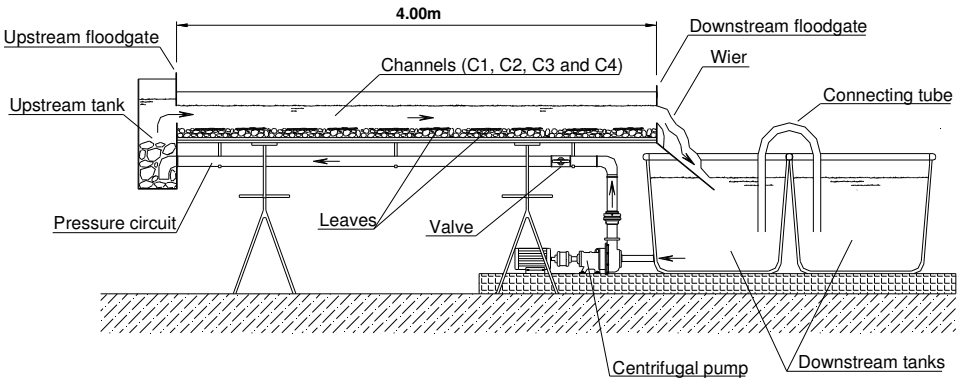
EXPERIMENT 1: Physical *vs.* shredder fragmentation in artificial channels

Artificial channels and current velocity

To assess the effects of current velocity and invertebrate activities in leaves, 4 acrylic contiguous artificial channels were used. The indoor channels had a total length of 4 m, width of 0.15 m (surface area (each)=0.6 m^2) and height of 0.20 m (**Fig. 1**). The channels ended in two 0.5 m^3 fiberglass reservoirs, to which a centrifuge pump was connected (**Fig. 1a**). The substrate was a mixture of stream gravel (2.5–15 cm size) and sand (0.9–2 mm grain size). The water used in the channels was collected from S. João stream (Lousã Mountain, Portugal; 40º05'59'' N, 8º14'02'' W) five days before the experiment started. By the middle of each experiment (day 8 or 15), 0.5 m^3 of stream water were added to compensate for evaporation. When collecting water from the stream, pH (Jenway 3310) and conductivity and temperature (WTW LF 330) were measured. In addition, 1 L of stream water was filtered (Millipore APFF), stored in acid washed plastic bottles, transported to the laboratory in ice, analyzed for alkalinity (by titration to an end point of 4.5; APHA, 1995) and a subsample frozen until analyzed for nutrients. Nitrogen was determined by ion chromatography (Dionex DX-120), and soluble reactive phosphorus (SRP) was determined by the ascorbic acid method (APHA, 1995)). Current velocity was also measured in several zones of the stream with a current meter (Valeport 15277).

As an *inoculum* of aquatic fungi, conditioned leaves (approximately 20 g) were collected from the stream and placed in fine mesh bags in the upstream tank that delivered water to the channels (**Fig. 1a**). Current velocity was set to match the maximum measured in zones of the stream where there was organic matter accumulation (0.20 m s^{-1}; channels C3 and C4) and to a low value (0.05 m s^{-1}; channels C1 and C2). Current velocity was regulated by acrylic barriers located upstream and downstream each channel (**Fig. 1a**).

1a



1b

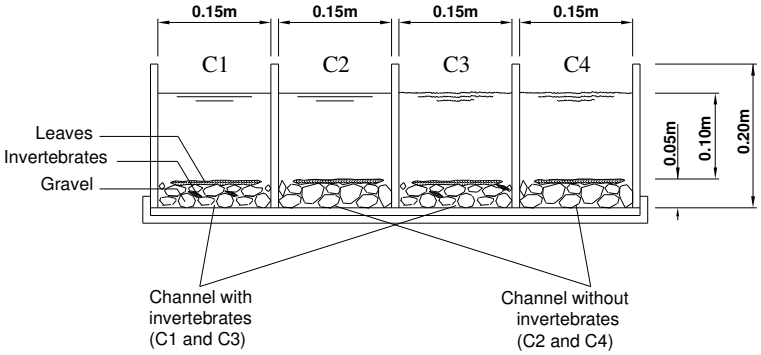


Figure 1. Scheme of the artificial channels used in the laboratory experiments in lateral (**a**) and cross-section (**b**) views. The arrows show the direction of flow in the hydraulic circuit. Channels C1 and C2 had low current velocity while channels C3 and C4 had high current velocity. Channels C1 and C3 had shredders.

Invertebrate shredders

Individuals of the shredder *Sericostoma* sp. (Trichoptera: Sericostomatidae) were added in one low (C1) and one high (C3) current velocity channel, at densities similar to the ones observed in S. João stream, i.e. 100 individuals per channel (initial individual dry mass=7.0±3.2 mg, mean±1SE). This species is generally present and abundant all over the year in the area. At our sampling site (S. João stream, Lousã Mountain), an annual mean of 6.3 g AFDM m^{-2} of coarse particulate organic matter (CPOM) and annual mean density of 119 sericostomatids m^{-2} were reported by GONZÁLEZ & GRAÇA (2003). The number of invertebrates in the channels was a compromise between the value expected in terms of area of each channel (71 individuals per 0.6 m^2) and the number expected per amount of organic

matter in each channel (138 individuals per 7.3 g AFDM; see below). The density used in the channels was a lower consumer/resource ratio than observed in the stream. Food was therefore assumed not to be a limiting factor.

The animals were in the laboratory for at least one week before being placed in the channels. In the laboratory they were maintained in plastic boxes, with aerated stream water and with stream sand in the bottom (0.9–2 mm grain size), exposed to a light-dark photoperiod of 12/12 h. During this time animals were fed with conditioned alder leaves.

Litter bags and decomposition

Alder (*Alnus glutinosa* (L.) GAERTNER) leaves were used as a decomposition substrate. Leaves were collected from the same group of trees in Varandas do Ceira (Portugal, 40º10'20'' N; 8º18'10'' W) just after abscission (4–12 November, 2002). They were air dried and stored dry until needed. Batches of 1±0.25 g of dry leaves (average per bag=0.85 g; average initial AFDM per bag=0.71 g) were rehydrated and placed in fine mesh (FM; 10 x 15 cm, 0.5 mm mesh size) and coarse mesh (CM; 10 x 15 cm, 10 mm mesh size) bags. Five fine mesh and 5 coarse mesh bags were placed in each of four artificial channels. Six bags were set apart to determinate initial ash free dry mass (AFDM).

One fine mesh and one coarse mesh bag were retrieved from each channel at days 3, 7, 14, 21, and 28 (these bags will be referred to as Set I). To maintain the same amount of organic matter in the channels, each retrieved bag was replaced with a new one. The replacement bags were taken all at once at day 31. So, they were in water for 3, 10, 17, 24 and 28 days (these bags will be referred to as Set II). To maintain the density of invertebrates, pupae and empty cases were replaced weekly.

To assess remaining mass, leaf remains were placed in an oven at 70 ºC for 72 h, weighed, ashed at 550 ºC for 4 h and reweighed to calculate AFDM. At each sampling date, conductivity, temperature and discharge (volumetric method) were determined and 1 L of channel water was collected for nutrient analysis and alkalinity determination (as described above). The experiment was repeated three times (the 1st run on February, the 2nd on March and the 3rd on April, 2003), to generate replicates. Between each run, water, conditioned leaves and animals were changed and stones, sand and channels were cleaned.

EXPERIMENT 2: Effects of high current on leaves in advanced stage of decomposition

Litter bags and decomposition

To assess the effects of increasing current velocity in mass loss, 52 fine mesh bags containing alder leaves (origin: Casa do Sal, Portugal, 40º6'5'' N; 8º13'49'' W; prepared as before) were placed in a low current stretch of the S. João stream, for 15 days to allow decomposition and softening. Bags were then transported in an ice chest to the laboratory. The leaves were gently rinsed with tap water and transferred to 20 coarse mesh (10 mm mesh size, to allow physical abrasion by water flowing) and 20 fine mesh (0.5 mm mesh size, to minimize the effect of current velocity) bags and placed in the channels (5 of each type in each channel), which contained tap water but not sediments or invertebrates. The 12 samples left were used to calculate the initial AFDM of leaves placed in the channels.

These bags were retrieved all at once after 15 days (therefore after 1 month of decomposition). Leaf remains were rinsed with tap water, dried (70 ºC for 72 h), weighed, ashed (550 ºC for 4 h) and reweighed to calculate AFDM. The experiment was repeated 4 times (between May and August, 2004) with increasing current velocities; the 1st run with 0.37–0.53 m s^{-1}, the 2nd run with 0.60–0.68 m s^{-1}, the 3rd run with 0.85–1.12 m s^{-1} and the 4th run with 1.14–2.35 m s^{-1} (determined by the volumetric method).

EXPERIMENT 3: Stream inter-habitat and temporal variability

Study site

Decomposition rates of alder leaves were also measured under natural conditions in S. João stream, in both summer (July 2003) and autumn (November/December 2004). S. João stream is a 4th order stream that drains a small siliceous catchment (18 km^2) with *Pinus pinaster*, *Castanea sativa*, *Eucalyptus globulus*, *Acacia dealbata*, *Populus* spp. and *Salix* spp. in the riparian zone. The stream substrate is mainly composed of gravel and pebbles, and sand in depositional zones.

Litter bags and decomposition

Alder leaves were collected from the same group of trees in Casa do Sal, in 12–15 June 2003, just after falling. Leaves were air dried and stored dry until needed. Batches of 2.75–2.85 g (summer) or 2.50–2.70 g (autumn) of dry leaves were rehydrated and allocated into coarse mesh bags. On June 28, 2003 (summer), six bags were placed in each of 18 stream sites classified into 6 classes (3 replicate sites per class) according to depth and current velocity

(**Table 1**). In autumn (start on November 13, 2004), only 12 stream sites classified into 4 classes were used (**Table 1**). On both dates, a group of six bags was taken to the stream and brought back to the laboratory to determinate initial AFDM, taking into account losses due to handling. One bag from each site (3 per class) was sampled after 2, 5, 12, 19, 26 and 33 (summer) or 8, 22, 29 and 35 (autumn) days in water and transported to the laboratory in individual zip lock bags. Conductivity, pH, temperature, depth and current velocity were measured using field meters; water was also taken for nutrient analysis as described above.

Table 1. Current velocity (m s^{-1}) and depth (m) (mean±1SD) in S. João stream, in summer (July, 2003) and autumn (Nov./Dec., 2004).

Site class		Current velocity		Depth
Summer, 2003				
LS	**L**ow	0.003 ± 0.002	**S**hallow	0.147 ± 0.010
LD	**L**ow	0.006 ± 0.002	**D**eep	0.367 ± 0.012
MS	**M**edium	0.388 ± 0.031	**S**hallow	0.168 ± 0.004
MD	**M**edium	0.318 ± 0.020	**D**eep	0.343 ± 0.015
HS	**H**igh	1.185 ± 0.117	**S**hallow	0.129 ± 0.010
HD	**H**igh	0.670 ± 0.057	**D**eep	0.343 ± 0.005
Autumn, 2004				
LS	**L**ow	0.142 ± 0.023	**S**hallow	0.098 ± 0.012
LD	**L**ow	0.145 ± 0.032	**D**eep	0.298 ± 0.011
HS	**H**igh	1.228 ± 0.197	**S**hallow	0.159 ± 0.009
HD	**H**igh	0.851 ± 0.162	**D**eep	0.391 ± 0.028

Macroinvertebrates from litter bags and benthos

Each sample was processed as previously described, except that invertebrates were collected on a 0.5 mm sieve and preserved in 70 % ethanol for later identification. Once during the experiment, benthic macroinvertebrates were sampled from each site, using a kick net (0.3 x 0.3 m opening and 0.5 mm mesh size; along 1 m, for 30 sec.). Samples were stored in 4 % formalin, sorted and invertebrates preserved in 70 % ethanol for later identification. Invertebrates were identified to genus or species, except for Oligochaeta (family), Hidracarina, Ostracoda and Nemathelmintha (presence) and some Diptera (subfamily or tribe), and classified into 2 groups: shredders and non-shredders (MERRIT & CUMMINS, 1996; TACHET *et al.*, 2000).

DATA ANALYSIS

EXPERIMENT 1: Physical *vs.* shredders fragmentation in artificial channels

Comparisons of water parameters of channels among the three experimental runs were made by 1-way ANOVA. When data were not normally distributed (Shapiro-Wilk's test) they were log transformed (ZAR, 1999).

Decomposition rates, *k*, were calculated by linear regression of ln transformed data (negative exponential model $M_t = M_o \cdot e^{-kt}$, where M_o is the initial mass, M_t is the remaining mass at time t, and *k* is the decomposition rate). For *k* per degree-day calculations, time (t) was substituted by the cumulative Celsius degrees at the sampling day. Slopes on ln-transformed data were compared by 5-way ANCOVA with run, mesh type, current velocity and shredders as categorical variables and time (or degree-days) as continuous variable.

EXPERIMENT 2: Effects of high current on leaves in advanced stage of decomposition

Mass loss (ln-transformed) of alder leaves incubated at different current velocities was compared by 3-way ANCOVA with run and mesh type as categorical variables and current velocity as continuous variable.

EXPERIMENT 3: Stream inter-habitat and temporal variability

Comparisons of stream water parameters between seasons were made by t test (ZAR, 1999). Decomposition rates (*k*) of alder leaves decomposing in S. João stream were calculated as before and related with current velocity by a linear regression model. Within each season, slopes on ln-transformed data were compared by 3-way ANCOVA with current and depth as categorical variables and time as continuous variable. For comparisons between seasons, slopes on ln-transformed data were compared by 4-way ANCOVA with season, current and depth as categorical variables and degree-days as continuous variable, followed by Tukey's test.

Comparisons of invertebrate abundance and richness among the different site classes in S. João stream were made by 2-way ANOVA with current and depth as categorical variables (benthic invertebrates) and 3-way ANOVA with current, depth and time as categorical variables (litter bag invertebrates). When data was not normally distributed (Shapiro-Wilk's test) they were log or log(x+1) transformed. Correspondence analysis (CA), on log(x+1) transformed invertebrate abundances, was performed for both litter bag and benthic samples collected in summer and autumn (CANOCO 4.5; TER BRAAK & SMILAUER, 1998).

Coordinates of samples on axis 1 and axis 2 were then correlated with current velocity (Spearman rank correlation, r). Statistical analyses were performed with STATISTICA 6 software unless otherwise indicated.

RESULTS

EXPERIMENT 1: Physical *vs.* shredders fragmentation in artificial channels

Water parameters

Conditions in all measured water parameters were not statistically different among the 3 experimental runs (1-way ANOVA, p>0.050). However, temperature, conductivity and nitrogen concentration were higher in the channels than in the stream whereas the opposite was true for phosphate. Alkalinity and pH were similar between channels and stream (**Table 2**).

Table 2. Channels and stream water chemical and physical parameters, over the three experimental runs (February–April, 2003). *n*, number of measurements.

	Channels			S. João stream		
	n	**mean**	**range**	***n***	**mean**	**range**
pH	8	7.1	6.9–7.4	5	6.9	6.7–7.2
Alkalinity (mg $CaCO_3$ L^{-1})	7	12.7	8.5–17.0	4	9.1	6.5–13.6
Conductivity (µS cm^{-1})	21	63.3	40.1–93.0	6	36.5	35.0–39.2
NO_3-N (µg L^{-1})	21	1270.9	246.7–3572.2	6	388.0	206.7–1164.7
SRP (µg L^{-1})	21	4.4	0–11.8	5	8.2	2.0–13.7
Temperature (ºC)	21	22.7	17.7–24.7	6	9.9	8.3–11.9

Current velocity in channels C1 and C2 was similar in all experimental runs (range=0.04–0.05 m s^{-1}). Similarly, in channels C3 and C4 current velocities ranged from 0.17 to 0.25 m s^{-1}. The ratio between the high current velocity and the low current velocity channels was always 5. In the stream, in places were there was organic matter accumulation, the mean current velocity was 0.13 m s^{-1} (±0.10), but it reached 1.94 m s^{-1} (±1.06) in places were there was no organic matter accumulation.

Invertebrate shredders

One hundred sericostomatids were placed in channels C1 and C3 at the beginning of each experimental run. However, due to pupation, and in spite of larvae replacement during the experiment, some animals were lost and by the end of each run the final number of

individuals in each channel varied between 46 and 71, being the difference between channels always ≤16 %.

Decomposition

Decomposition rates in channels were measured in two sets of leaves: leaves retrieved periodically from the channels (Set I) and leaves placed periodically in the channels and retrieved all at once (Set II). Leaves in coarse mesh bags lost 65 (Set I)–69 (Set II) % of their initial AFDM after 28 days in low current+shredders channel, and 73 (Set I)–75 (Set II) % in high current+shredders channel. All the other mesh types and channels had higher remaining leaf material after 28 days (58–67 %) (**Fig. 2** and **Table 3**). Decomposition rates were significantly affected by shredder presence (5-way ANCOVA, $p<0.001$) but not by current velocity (5-way ANCOVA, $p>0.132$) (**Table 4**). In the presence of shredders (C1 and C3), decomposition rates were significantly higher in coarse mesh bags than in fine mesh bags (5-way ANCOVA, $p<0.001$; **Table 4**).

Table 3. Decomposition rates of alder leaves incubated in the channels (February–April, 2003), calculated on a per-day and on a per degree-day basis. Bag types with the same letter do not have significantly different *k* values (5-way ANCOVA, p>0.05).

			Set I		Set II	
Mesh type	**Shredders**	**Current**	***k* day^{-1}**	***k* degree-day^{-1}**	***k* day^{-1}**	***k* degree-day^{-1}**
Coarse	Yes	Low	0.0368^{ac}	0.0016^{ab}	0.0416^{a}	0.0017^{a}
Fine	Yes	Low	0.0191^{b}	0.0008^{b}	0.0187^{b}	0.0008^{b}
Coarse	No	Low	0.0210^{bc}	0.0009^{b}	0.0182^{b}	0.0008^{b}
Fine	No	Low	0.0213^{bc}	0.0009^{b}	0.0166^{b}	0.0007^{b}
Coarse	Yes	High	0.0472^{a}	0.0020^{a}	0.0455^{a}	0.0019^{a}
Fine	Yes	High	0.0215^{bc}	0.0009^{b}	0.0212^{b}	0.0009^{b}
Coarse	No	High	0.0219^{bc}	0.0009^{b}	0.0184^{b}	0.0008^{b}
Fine	No	High	0.0238^{bc}	0.0010^{b}	0.0198^{b}	0.0008^{b}

EXPERIMENT 2: Effects of high current on leaves in advanced stage of decomposition

Decomposition

After 15 days in the stream, leaves lost around 50 % of their initial mass, primarily due to tissue softening by microorganisms since almost no invertebrates (except for some chironomid early stage larvae) were found inside mesh bags and no reduction in area was noticed. The remaining mass was considered the initial mass for channels experiment. After 15 days in the channels, there was still 47 (at 0.8 m s^{-1})–99 (at 0.37, 0.53 and 2.35 m s^{-1}) % of mass remaining (**Fig. 3**). No effect of current velocity or mesh type was detected on % mass remaining (3-way ANCOVA, p=0.103 and 0.217, respectively; **Table 5**). Even when the channel with current of 2.35 m s^{-1} was not considered in the analysis, as it seemed to be an

outlier, current velocity and bag type were considered to be unimportant to mass loss of alder leaves (3-way ANCOVA, p=0.868 and 0.258, respectively; **Table 5**). However, as runs were carried out at different times, and without replication, a significant effect of the run was detected (3-way ANCOVA, p<0.001; **Table 5**).

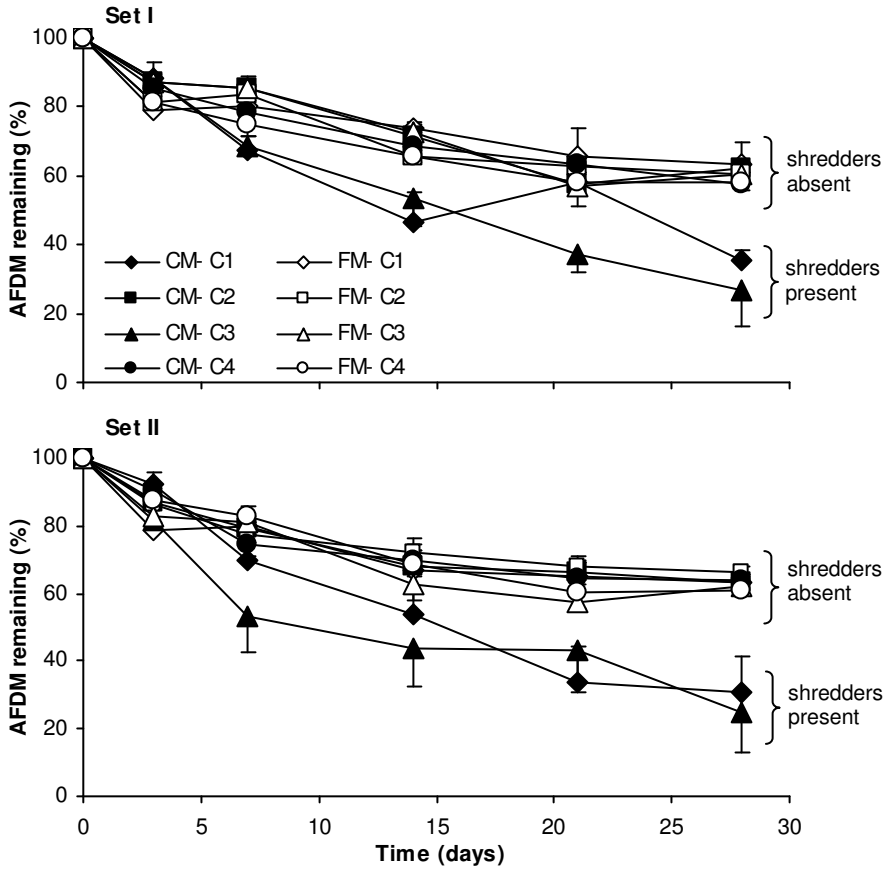


Figure 2. Remaining mass (mean±1SE) of alder leaves (two sets: see text) incubated in coarse mesh (CM) and fine mesh (FM) bags in channels 1 (C1: low current+shredders), 2 (C2: low current-shredders), 3 (C3: high current+shredders) and 4 (C4: high current-shredders).

EXPERIMENT 3: Stream inter-habitat and temporal variability

Water parameters

Water in S. João stream was circumneutral, with low conductivity and low nutrient content, in both summer 2003 and autumn 2004. As expected, temperature was significantly lower in autumn (8.2 ºC) than in summer (16.4 ºC; t test, p<0.001; **Table 6**). Current velocity and depth did not vary greatly in the experimental sites during the experiment (**Table 1**).

Decomposition

Litter decomposition proceeded much faster in summer than in autumn (**Fig. 4**). After the 1st week in water, leaves incubated in summer had already lost 23–30 % of their initial AFDM while leaves incubated in autumn only lost 6–13 % of their initial AFDM, being this loss primarily due to leaching. After 5 weeks in water, only 12–22 % of the initial AFDM was still remaining in bags incubated in summer while 25–63 % was still left in bags incubated in autumn (**Fig. 4**).

Table 4. Results from the 5-way ANCOVA (run, current velocity, shredders and mesh type as categorical variables; time as continuous variable) on mass loss of alder leaves incubated in the artificial channels.

		Set I		Set II	
Effect	**df**	**F ratio**	**p**	**F ratio**	**p**
Intercept	1	707.268	<0.001	441.853	<0.001
Time	1	168.294	<0.001	118.430	<0.001
Run	2	1.428	0.490	5.685	0.058
Cvel	1	1.554	0.213	2.265	0.132
Shredders	1	12.552	<0.001	36.682	<0.001
Mesh type	1	15.472	<0.001	24.512	<0.001
Run*Cvel	2	0.793	0.673	5.842	0.054
Run*Shredders	2	3.581	0.167	0.030	0.985
Cvel*Shredders	1	0.010	0.920	1.258	0.262
Run* Mesh type	2	5.361	0.069	0.978	0.613
Cvel* Mesh type	1	0.529	0.467	0.887	0.346
Shredders* Mesh type	1	28.972	<0.001	22.964	<0.001
Run*Cvel*Shredders	2	2.006	0.367	6.367	0.041
Run*Cvel* Mesh type	2	0.006	0.997	4.780	0.092
Run*Shredders* Mesh type	2	3.978	0.137	0.194	0.908
Cvel*Shredders* Mesh type	1	1.105	0.293	1.769	0.184
Run*Cvel*Shredders* Mesh type	2	1.494	0.474	5.408	0.067

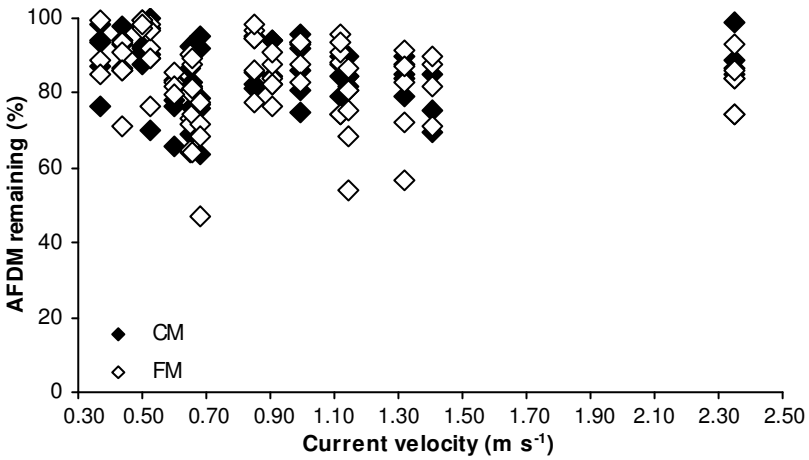


Figure 3. Remaining mass of alder leaves incubated in coarse mesh (CM) and fine mesh (FM) bags in artificial channels with increasing current velocity.

Table 5. Results from the 3-way ANCOVA (run and mesh type as categorical variables; current velocity as continuous variable) on mass loss of alder leaves in advanced stage of decomposition incubated in the artificial channels.

Effect	df	F ratio	p	p*
Intercept	1	516.159	<0.001	<0.001
Cvel	1	2.656	0.103	0.868
Run	3	57.269	<0.001	<0.001
Mesh type	1	1.521	0.217	0.258
Run* Mesh type	3	5.151	0.161	0.247

p, considering all current velocities; p*, without considering 2.35 m s^{-1}

Table 6. Stream water chemical and physical parameters in summer (July, 2003) and autumn (Nov./Dec., 2004). *n*, number of measurements.

	Summer, 2003			Autumn, 2004		
	n	mean	range	*n*	mean	range
pH	7	7.1	7.0–7.3	5	6.7	6.6–6.7
Conductivity (µS cm^{-1})	7	47	44–50	5	37	36–37
NO_3-N (µg L^{-1})	8	179.3	141.0–220.0	5	157.0	138.8–168.6
SRP (µg L^{-1})	6	10.3	6.4–14.5	5	12.4	10.6–13.6
Temperature (ºC)	7	16.4	15.3–18.0	5	8.2	7.5–9.8

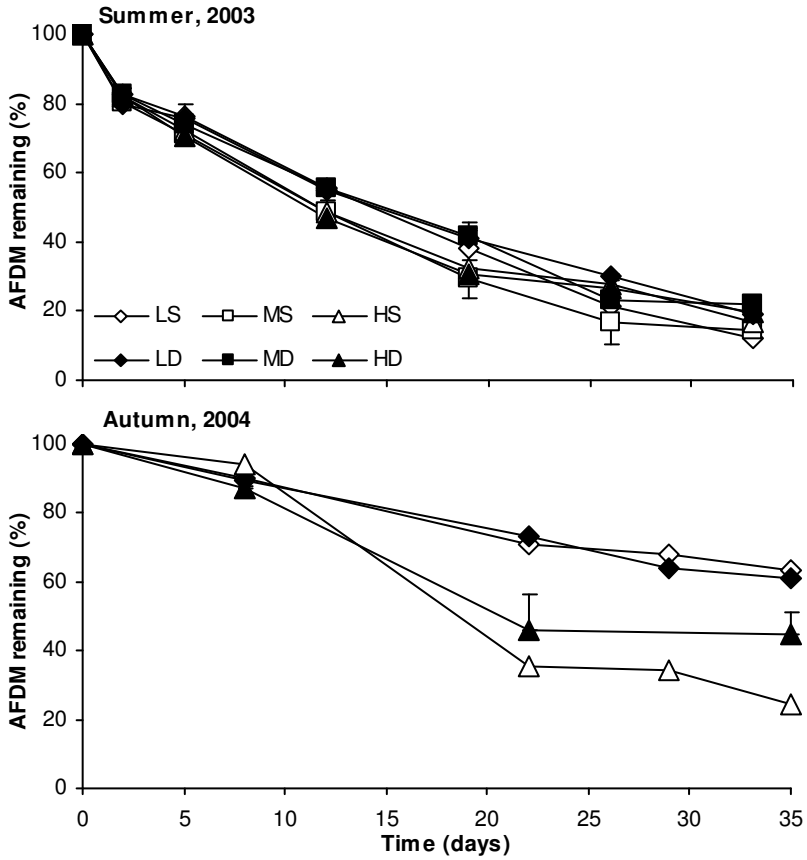


Figure 4. Remaining mass (mean±1SE) of alder leaves incubated in S. João stream, in summer (July, 2003) and autumn (Nov./Dec., 2004). See Table 1 for class definitions.

In summer, decomposition rates varied between 0.0489 d^{-1} and 0.0645 d^{-1}, but there were no significant differences among site classes (3-way ANCOVA, p=0.125 for current velocity and 0.109 for depth; **Table 7** and **8**). However, in autumn, decomposition rates varied between 0.0136 d^{-1} and 0.0417 d^{-1} and significant differences were found between low current velocity and high current velocity site classes (3-way ANCOVA, p<0.001; **Table 7** and **8**).

Table 7. Decomposition rates of alder leaves incubated in S. João stream (see Table 1 for class definitions), calculated on a per-day and on a per degree-day basis. *, within each season, bag types with the same letter do not have significantly different *k* values (3-way ANCOVA, p>0.050). **, considering both seasons, bag types with the same letter do not have significantly different *k* values (4-way ANCOVA, p>0.050).

Site class	*k* day^{-1}*	R^2	*k* degree-day^{-1}**	R^2
Summer, 2003				
LS	0.0598^{a}	0.9395	0.0035^{ab}	0.9308
LD	0.0645^{a}	0.8548	0.0028^{a}	0.9560
MS	0.0546^{a}	0.9256	0.0037^{ab}	0.8482
MD	0.0489^{a}	0.9664	0.0029^{a}	0.9181
HS	0.0497^{a}	0.9267	0.0032^{a}	0.9223
HD	0.0541^{a}	0.8326	0.0031^{a}	0.8309
Autumn, 2004				
LS	0.0136^{a}	0.9604	0.0017^{a}	0.9271
LD	0.0147^{a}	0.7077	0.0018^{a}	0.7023
HS	0.0417^{b}	0.6032	0.0051^{b}	0.5929
HD	0.0270^{b}	0.5314	0.0033^{ab}	0.5245

Although decomposition rates on a per day basis were much higher in summer than in autumn, comparisons between seasons must be done using decomposition rates on a per degree-day basis to account for differences in temperature between seasons. Significant differences were found between high current velocity-shallow site class in autumn (HS, 2004) and low current velocity site classes in autumn (LS and LD, 2004), deep site classes in summer and high current velocity site classes in summer (LD, MD, HD and HS, 2003) (4-way ANCOVA, p<0.001; **Table 7** and **8**). No relationship between decomposition rates of alder leaves and current velocity was found in summer (linear regression, p=0.976, R^2<0.001), however, in autumn, decomposition rates were significantly related to current velocity (linear regression, p=0.017, R^2=0.97).

Table 8. Results from the 3-way (current velocity and depth as categorical variables; time as continuous variable) and 4-way ANCOVA (season, current velocity and depth as categorical variables; degree-days as continuous variable) on mass loss of alder leaves incubated in different site classes in S. João stream in summer and autumn.

	Summer, 2003		
Effect	**df**	**F ratio**	**p**
Intercept	1	924.171	<0.001
Time	1	1239.243	<0.001
Cvel	2	4.153	0.125
Depth	1	2.574	0.109
Cvel*Depth	2	4.425	0.109
	Autumn, 2004		
Effect	**df**	**F ratio**	**p**
Intercept	1	38.965	<0.001
Time	1	42.672	<0.001
Cvel	1	17.292	<0.001
Depth	1	0.856	0.355
Cvel*Depth	1	1.241	0.265
	Summer *vs.* Autumn		
Effect	**df**	**F ratio**	**p**
Intercept	1	360.040	<0.001
Degree-days	1	478.384	<0.001
Season	1	17.326	<0.001
Cvel	1	28.597	<0.001
Depth	1	1.185	0.276
Season*Cvel	1	13.936	<0.001
Season*Depth	1	0.874	0.350
Cvel*Depth	1	0.861	0.354
Season*Cvel*Depth	1	2.267	0.132

Macroinvertebrates from litter bags and benthos

Between 60 (autumn) and 65 (summer) taxa were recovered from the stream benthos. Shredders corresponded to 12 (summer)–14 % (autumn) of individuals. Leuctridae (Plecoptera) was the most abundant group (accounting for more than 50 % of total number of shredders in 61 % of sites) on summer, and Nemouridae (Plecoptera) the most abundant (accounting for more than 50 % of total number of shredders in 75 % of sites) on autumn. Generally, sites did not differ in terms of abundance and richness total invertebrates and shredder (2-way ANOVA, p>0.050), except high current sites in autumn that had significantly higher shredder richness than low current sites (2-way ANOVA, p=0.030) (**Fig. 5** and **Table 9**).

Between 26 (autumn) and 60 (summer) taxa were recovered from litter bags. Shredders accounted for 7 (summer)–40 % (autumn) of the total number of individuals being *Lepidostoma hirtum* (Lepidostomatidae, Trichoptera) the most abundant shredder in both seasons. In summer, the abundance of total invertebrates and shredder generally increased in

leaf material up to day 26 (**Fig. 6a**) while the richness of total invertebrates and shredder increased along the experiment with higher values in the last sampling date (**Fig. 6b**). In autumn, invertebrates colonized litter bags only after 22 days of submersion, and their abundance and richness increased along the experiment until the last sampling day (**Fig. 6c** and **d**). In summer, medium current sites had higher abundance and richness than low and high current sites (3-way ANOVA, $p<0.001$; **Table 10**), this being particularly evident by day 26. In autumn, no significant differences were found among site classes for the 4 invertebrate parameters (3-way ANOVA, $p>0.050$; **Table 10**).

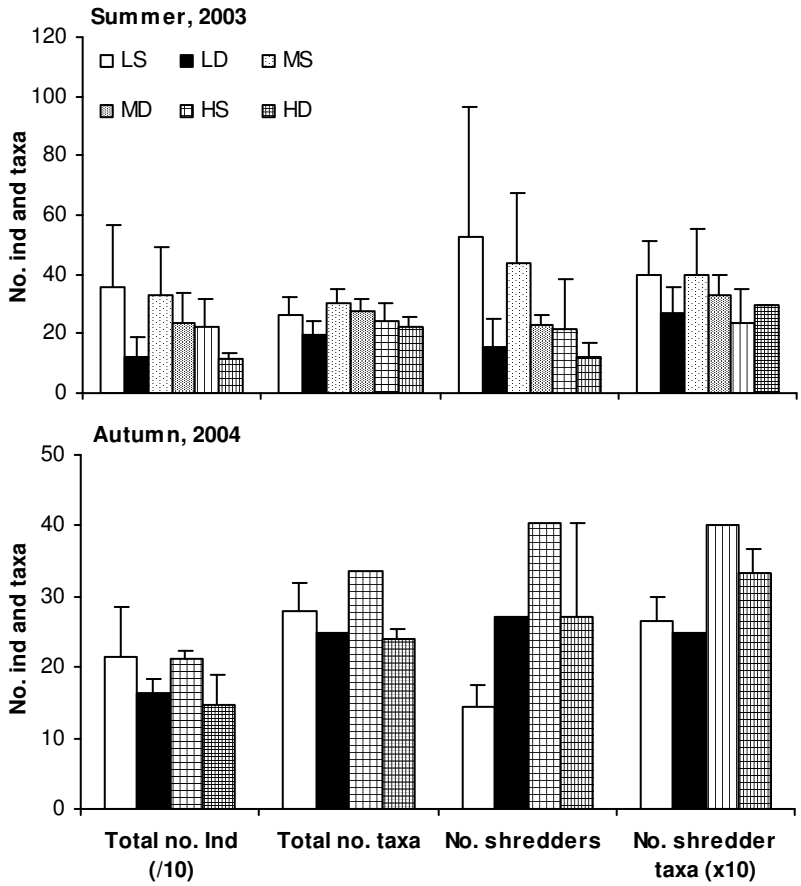


Figure 5. Abundance and richness of benthic total invertebrates and shredder (mean±1SE) in S. João stream, in summer (July, 2003) and autumn (Nov./Dec., 2004). See Table 1 for class definitions.

Table 9. Results from the 2-way ANOVA (current velocity and depth as categorical variables) on the abundance and richness of benthic invertebrates in different site classes in S. João stream in summer and autumn.

	Total abundance	Total richness	Shredder abundance	Shredder richness
Summer, 2003				
Intercept	<0.001	<0.001	<0.001	<0.001
Cvel	0.512	0.402	0.453	0.609
Depth	0.186	0.458	0.651	0.982
Cvel*Depth	0.686	0.848	0.756	0.482
Autumn, 2004				
Intercept	<0.001	<0.001	<0.001	<0.001
Cvel	0.860	0.488	0.319	0.030
Depth	0.369	0.081	0.976	0.340
Cvel*Depth	0.627	0.283	0.138	0.614

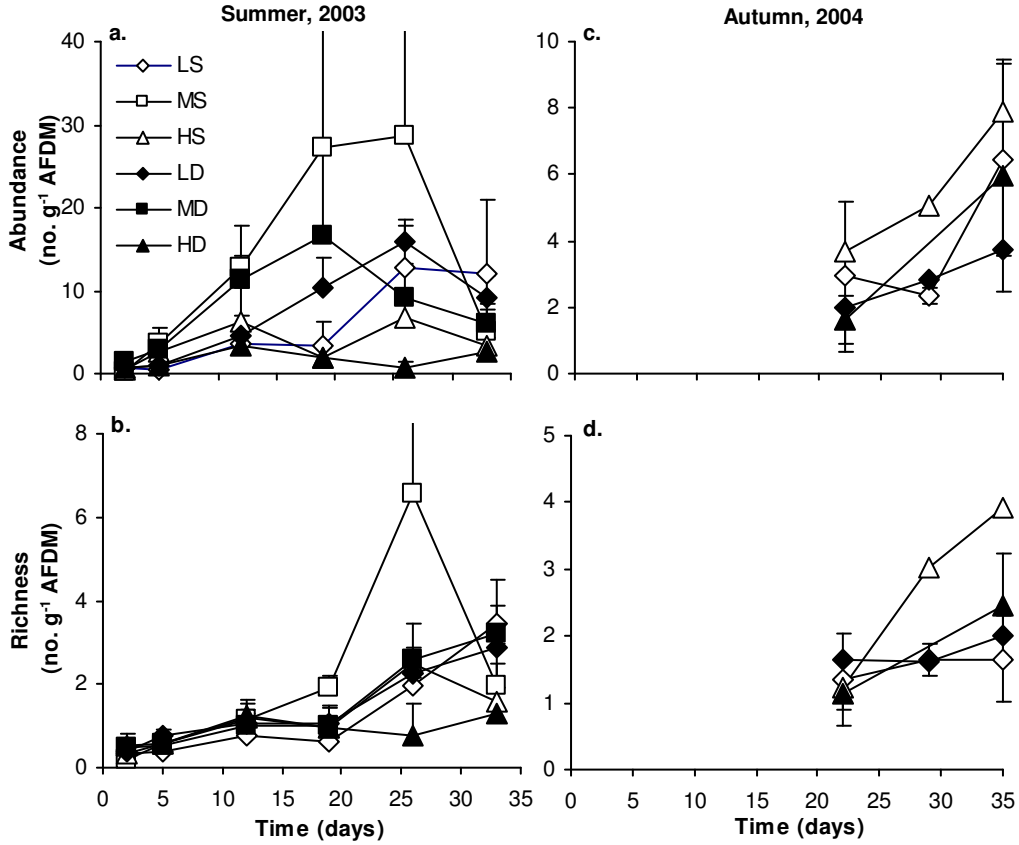


Figure 6. Abundance (**a** and **c**) and richness (**b** and **d**) of shredders (mean±1SE) colonizing alder leaves incubated in S. João stream, in summer (July, 2003) and autumn (Nov./Dec., 2004). The same pattern exists for abundance and richness of total invertebrates. See Table 1 for class definitions.

Table 10. Results from the 3-way ANOVA (current velocity, depth and time as categorical variables) on abundance and richness of invertebrates colonizing alder leaves incubated in different site classes in S. João stream in summer and autumn.

	Total abundance	Total richness	Shredder abundance	Shredder richness
Summer, 2003				
Intercept	<0.001	<0.001	<0.001	<0.001
Cvel	<0.001	<0.001	<0.001	0.108
Depth	0.153	0.343	0.916	0.664
Time	<0.001	<0.001	<0.001	<0.001
Cvel*Depth	0.112	<0.001	0.115	0.495
Cvel*Time	<0.001	0.046	0.053	0.188
Depth*Time	0.391	0.441	0.326	0.336
Cvel*Depth*Time	0.475	0.729	0.867	0.711
Autumn, 2004				
Intercept	<0.001	<0.001	<0.001	<0.001
Cvel	0.147	0.071	0.846	0.655
Depth	0.081	0.207	0.534	0.945
Time	0.045	0.031	0.082	0.073
Cvel*Depth	0.120	0.105	0.833	0.466
Cvel*Time	0.778	0.368	0.710	0.209
Depth*Time	0.349	0.128	0.660	0.534
Cvel*Depth*Time	0.757	0.868	0.992	0.545

When correspondence analysis was applied to the macroinvertebrate communities, from both stream benthos and litter bags, a continuous distribution of samples along a current velocity gradient (axis 1) was observed in both seasons (Spearman rank correlation; $p<0.001$, $r=0.85$ (benthic samples) and $r=0.74$ (litter bag samples) in summer; $p=0.049$, $r=0.59$ (benthic samples) and $p<0.001$, $r=0.79$ (litter bag samples) in autumn; **Fig. 7**).

DISCUSSION

Our global objective was to assess the relative importance of current velocity in the decomposition of leaves. If current is a potential source of variability, then many results of decomposition studies could be influenced by the places where leaves are located during the experiments. This is particularly important if decomposition rates are going to be used as a functional measure of stream health condition (PASCOAL *et al.*, 2001, 2003; GESSNER & CHAUVET, 2002; DANGLES *et al.*, 2004).

In the experimental channels, the presence of invertebrates caused a significant increase in mass loss of leaves (k CM bags > k FM bags), as predicted from the literature (e.g. CUFFNEY *et al.*, 1990; STEWART, 1992), whereas in experimental channels with no invertebrates, k values for leaves in for coarse and fine mesh bags were similar, regardless

current velocity. For fine mesh bags there was no difference in *k* values among the 4 channels. The absence of an effect of current velocity was probably due to the low range of values tested (0.05–0.2 m s^{-1}). Nevertheless, results from the channels experiments suggest therefore that for current velocity values up to 0.20 m s^{-1}, physical fragmentation can be considered unimportant in decomposition experiments, while shredder presence is a major factor controlling decomposition.

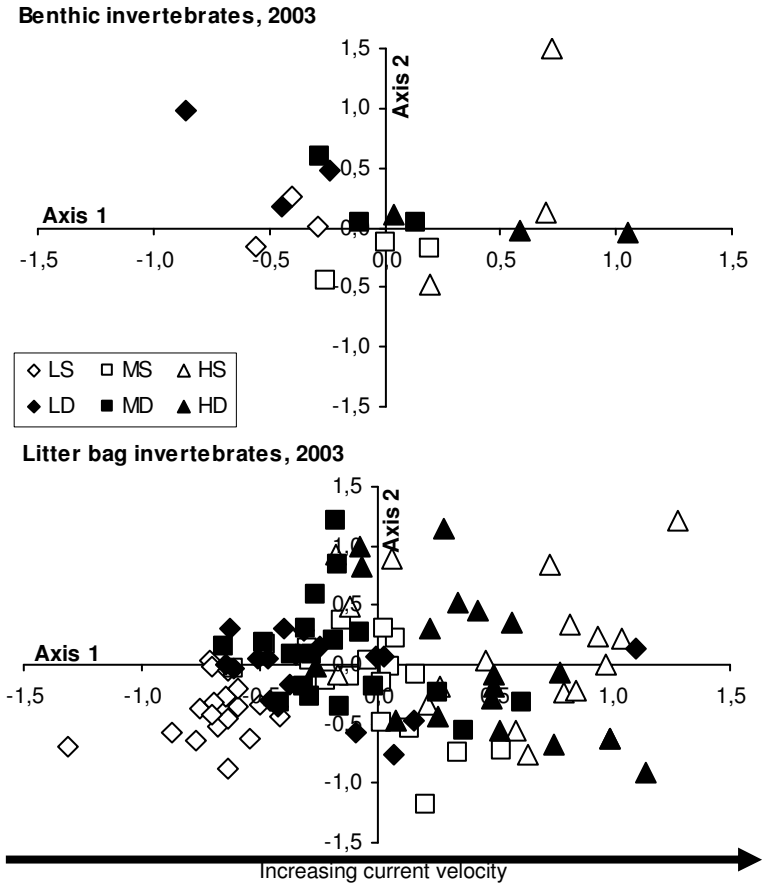


Figure 7. Ordination (CA) of benthic and litter bag invertebrates samples collected from S. João stream in summer 2003 (axis 1+2 explained 15.0 % and 28.9 % of the variability, respectively). Correspondence analysis using invertebrate samples collected in autumn 2004 gave similar results with axis 1+2 explaining 26 % and 37.3 % of the variability for benthic and litter bag invertebrates, respectively. See Table 1 for class definitions.

The *k* values obtained in the channels experiment (0.0166 d^{-1}–0.0472 d^{-1}) were higher than the ones obtained in other studies for the same leaf species, in Portugal (CANHOTO & GRAÇA, 1996; ABELHO, 2001) and in other European countries (CHERGUI & PATTEE, 1990; GESSNER *et al.*, 1991; BALDY & GESSNER, 1997; HIEBER & GESSNER, 2002), or similar leaves

(GESSNER *et al.*, 1998; ROBINSON & GESSNER, 2000; ABELHO, 2001). These differences could be explained by higher water temperature observed in this study since it has been reported that high temperatures enhance leaching (CHERGUI & PATTEE, 1990), decomposition (CHERGUI & PATTEE, 1990; IRONS *et al.,* 1994; JONSON *et al.*, 2001) and fungal growth and sporulation (GRAÇA & FERREIRA, 1995; CHAUVET & SUBERKROPP, 1998).

It may be argued that current may be more important in the late stages of decomposition when leaves are more fragile due to the digestion of tissues by microbial enzymes (e.g. CHERGUI & PATTEE, 1988; CANTON & MARTINSON, 1990). However, in spite of the high current velocity values tested (up to 2.35 m s^{-1}; experiment 2), mass loss by alder leaves was not related to current velocity and, once again, physical fragmentation was unimportant. Although there is no doubt that current should have an effect on the fragmentation of leaves in natural packs, it is plausible that leaves inside bags are unexposed to the direct effect of current. If this is the case, the fine-coarse mesh bag approach does indeed quantify the difference between invertebrate and microbial decomposition, but our ability to predict decomposition rates using this approach underestimates what happens under realistic field conditions.

In the field experiment carried out during summer no significant differences in *k* values (k=0.0489 d^{-1} –0.0645 d^{-1}) were observed among site classes differing in current velocity (range: 0.003–1.185 m s^{-1}) and depth (0.13–0.37 m). This absence of an effect of current velocity in litter decomposition was not a result of a compensation effect by invertebrate activity, in spite of differences in invertebrate numbers between different current velocities, as there were not higher numbers in low current and low numbers in high current velocity site classes. In autumn, however, alder leaves incubated in high current velocity site classes decomposed at higher rate than leaves in low current velocity site classes. Since current velocity values were not much different between summer and autumn, for the same site class, the difference observed in autumn could be due to an indirect effect related to a higher amount of transported sediments in the water during this season, caused by rains (absent in summer). The sediments could act as abrasive agents on the leaves. HEARD *et al.* (1999) found mechanical abrasion to be an important contributor to organic matter processing in streams, though they evaluated the effect of coarse sediments. Another explanation for the difference could be the higher shredder diversity observed in fast flowing-shallow stream sectors, which is consistent with previous studies indicating that, at river stretch level, shallow riffles had

significantly higher coarse particulate organic matter and invertebrates, including shredders, than deeper sections (GRAÇA *et al.*, 2004).

The faster decomposition in summer (k=0.0489 d^{-1} –0.0645 d^{-1}) than in autumn (k=0.0136 d^{-1} –0.0417 d^{-1}) could be attributed to the temperature stimulation effect on leaching (CHERGUI & PATTEE, 1990) and on microbial activity (e.g. GRAÇA & FERREIRA, 1995; CHAUVET & SUBERKROPP, 1998). Also, in summer there is usually a food limitation for invertebrate shredders as much of the autumnal litter input was decomposed/washed downstream; the litter bags acted in this situation as food islands on which invertebrates probably fed at higher rates than in autumn when there is no food limitation. When the data was expressed on a per degree-day basis no differences were found between seasons; only high current-shallow site class (HS, 2004) was significantly different from the majority of other site classes.

Our results differ from other studies. CHERGUI & PATTEE (1988) reported a fast decomposition rate of poplar leaves at high current velocity (0.44 m s^{-1}) than in stagnant water, mostly as a consequence of microbial and invertebrate feeding. CANTON & MARTINSON (1990) reported higher mass loss of willow leaves in artificial channels with higher (0.26 and 0.31 m s^{-1}) than with low (0.12 and 0.19 m s^{-1}) current velocity, but only after 6 weeks. Finally, VINGADA (1995), using alder leaves also observed higher decomposition for coarse and fine mesh bags in an artificial channel with high current velocity (0.53 m s^{-1}) than in a tank with stagnant water.

The importance of invertebrates in decomposition is also unclear. RADER *et al.* (1994) using chemical inhibitors to isolate the effect of current velocity and invertebrate activity concluded that neither base-flow current velocity nor shredders were important in the decomposition of sweet gum leaves and concluded that microbial degradation was the dominant factor controlling decomposition. On the other hand, HIEBER & GESSNER (2002) found that invertebrates were responsible for 64 % of mass loss in alder leaves. CUFFNEY & WALLACE (1989) reported that in the absence of invertebrates (by excluding them with insecticide) the amount of CPOM accumulating in a stream reach increased because it was not being decomposed, which resulted in a decrease of the amount of FPOM exported. Other studies showed lower decomposition rates of leaves in streams with low number of invertebrates when compared to streams with high densities of invertebrates (CUFFNEY *et al.*, 1990; STEWART, 1992; FABRE & CHAUVET, 1998).

Generally, no significant differences in invertebrate numbers were found among site classes. Nevertheless, when considering the invertebrate community, both from litter bags and benthos, it was possible to notice a shift in the community along a current velocity gradient. This was caused by some taxa with current velocity preferences which avoid sites where current velocity was not adequate to their life style; e.g. *Rhitrogena* sp. (Heptageniidae), a dorso-ventral compressed mayfly, and simulids (filter dipterans) were almost absent from low current velocity sites.

In conclusion, this study showed that current velocity (up to 2.35 m s^{-1}), by itself, did not affect the decomposition rates of alder leaves. However, the presence of fine sediments in water had the potential to amplify the effect of current velocity. On the other hand, unlike current, the presence of shredders increased the rate of mass loss of alder leaves.

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CHAPTER II

NUTRIENT ENRICHMENT OF STREAMS AFFECTS LITTER DECOMPOSITION AND ASSOCIATED FUNGI AND INVERTEBRATES

LEAF LITTER DECOMPOSITION AS A FUNCTIONAL PARAMETER TO ASSESS EUTROPHICATION IN STREAMS

GULIS V., FERREIRA V. & GRAÇA M.A.S. 2006. Stimulation of leaf litter decomposition and associated fungi and invertebrates by moderate eutrophication: implications for stream assessment. *Freshwat Biol* 51: 1655–1669

Photos:

Catelões River, Múceres, Caramulo Mountain, Portugal

Candal Stream, Candal, Lousã Mountain, Portugal

NUTRIENT ENRICHMENT OF STREAMS AFFECTS LITTER DECOMPOSITION AND ASSOCIATED FUNGI AND INVERTEBRATES

LITTER DECOMPOSITION AS A FUNCTIONAL PARAMETER TO ASSESS NUTRIENT ENRICHMENT IN STREAMS

ABSTRACT

Many human activities (e.g. agriculture, urban pollution) result in increases in nutrient concentrations in water bodies. Here it was evaluated the use of litter decomposition rates as a functional tool to assess the health of the aquatic ecosystem. To fulfill this objective, 5 reference and 5 nutrient enriched streams were compared in terms of (a) decomposition rates of alder and oak leaves, (b) abundance and richness of total invertebrates and shredders colonizing decaying leaves and (c) structural parameters and biotic indices based on the benthic macroinvertebrate community. In addition, one reference and one nutrient enriched stream were compared in terms of fungal biomass and sporulation and species richness of aquatic hyphomycetes associated with leaves. There was a tendency for higher decomposition rates in nutrient enriched than in reference streams, but this was significant only for oak. Decomposition rates of both alder and oak leaves increased with both nitrogen (up to 1.5 mg L^{-1}) and SRP (up to 60 μg L^{-1}) concentration in stream water. Abundance and richness of invertebrates and fungal biomass and sporulation were generally higher in the nutrient enriched than in the reference stream. The ratio of enriched/reference streams *k* (decomposition rate) value for oak leaves in coarse mesh bags classified all 5 enriched streams as impacted, according to the classification proposed by GESSNER & CHAUVET (2002). Among benthic structural parameters, only the biotic index BMWP' and 14 metrics (out of 45) discriminated between reference and nutrient enriched streams; from these, 10 were correlated with NO_3-N and 12 with SRP concentration in water. Multivariate analysis of invertebrate assemblages discriminated enriched from reference streams, with streams displaced along an SRP gradient. These results show that decomposition rates, especially those of oak leaves in coarse mesh bags, were useful in assessing stream health, with results comparable with those of multivariate analysis.

Key-words: alder, aquatic hyphomycetes, ecosystem functioning, oak, water quality

INTRODUCTION

Allochthonous organic matter provided by trees from the riparian zone is an important source of energy in small woodland streams where shading limits primary production (VANNOTE *et al*., 1980; ABELHO, 2001). The decomposition of this organic matter is carried out by microorganisms, mainly fungi (BALDY *et al*., 1995; HIEBER & GESSNER, 2002; GULIS & SUBERKROPP, 2003a,b), and invertebrate shredders (STEWART, 1992; ROBINSON *et al*. 1998; GRAÇA, 2001).

As fungi can obtain nutrients from both the substrate and surrounding water (SUBERKROPP, 1998), both leaf quality (ESCUDERO *et al*., 1991; BALDY *et al*., 1995; CHADWICK & HURYN, 2003) and nutrient concentration in water (SUBERKROPP & CHAUVET, 1995; ROBINSON & GESSNER, 2000; GULIS & SUBERKROPP, 2003b) affect decomposition rates. Higher nutrient (N and/or P) availability has been shown to stimulate the activities of both microbes (SUBERKROPP & CHAUVET, 1995; GULIS & SUBERKROPP, 2003b; NIYOGI *et al*., 2003) and invertebrates (ROBINSON & GESSNER, 2000; ROSEMOND *et al*., 2002; NIYOGI *et al*., 2003; PASCOAL *et al*., 2003) that are generally translated into higher decomposition rates. This stimulation is usually higher for low quality substrates where the microbial community is probably nutrient limited (CHADWICK & HURYN, 2003; GULIS & SUBERKROPP, 2003b). However, at extremely high concentrations, nutrients in water can become inhibitory (SRIDHAR & BÄRLOCHER, 1997; SUBERKROPP, 1998), and hence leading to a reduction of decomposition rates. Also, associated with eutrophication there might be toxic substances (LECERF *et al*., 2006), sedimentation (NIYOGI *et al*., 2003) and oxygen depletion (RAVIRAJA *et al*., 1998) which can have negative effects on the aquatic communities and ecosystem functioning.

Presently, ecological integrity of aquatic ecosystems is being assessed using structural parameters primarily of the benthic macroinvertebrate community (ARMITAGE *et al*., 1983; RESH *et al*., 1995; GRAÇA & COIMBRA, 1998; JÁIMEZ-CUÉLLAR *et al*., 2004; review by METCALFE, 1989) and periphyton (ALMEIDA & GIL, 1998), but also of the fish (EMERY *et al*., 2003) and macrophyte communities (FERREIRA *et al*., 2005). However, ecosystem ecological integrity is composed of two components: structural integrity and functional integrity (GESSNER & CHAUVET, 2002). So, to fully assess the ecological integrity of ecosystems both structural and functional parameters should be monitored. Litter decomposition rates, as a key ecosystem-level process, have been suggested to be used as a functional tool to assess the aquatic ecosystem integrity, as a complement to structural measurements (PASCOAL *et al*.,

2001, 2003; GESSNER & CHAUVET, 2002). Litter decomposition is an integrative process linking the riparian zone, as the source of the organic matter and shading, and a diverse array of decomposers including fungi, bacteria and detritivores. Decomposers convert allochthonous organic matter into microbial biomass, and enhance the quality of leaves to invertebrates. The rate at which decay proceeds reflects the overall response of the community to abiotic characteristics of the environment, including anthropogenic stress (GESSNER *et al*., 1999; GESSNER & CHAUVET, 2002).

The objective of this study was to evaluate the potential for leaf litter decomposition rates, and associated fungal and invertebrate parameters, to be used as a tool to assess nutrient enrichment in streams. For this, 5 reference and 5 nutrient enriched streams were compared in terms of decomposition rates of alder and oak leaves, and other traditional structural parameters based on the benthic macroinvertebrate community.

METHODS

Study area

Five reference (R) and 5 nutrient enriched (N) streams were selected in two mountain areas in Central Portugal (Caramulo and Lousã mountains), with altitude ranging from 210 to 805 m a.s.l. (**Table 1**). The streams ranged from 2nd to 5th order, with catchment areas between 0.5 and 43.1 km^2, located in granitic or schistous areas (**Table 1**). Native deciduous trees (oak, *Quercus robur* (L.), chestnut, *Castanea sativa* MILL., and alder, *Alnus glutinosa* (L.) GAERTNER) shaded all streams. Streams were paired, each pair having a reference and a nutrient enriched stream chosen to be as contrasting as possible with respect to nutrient content (**Table 2**).

Water parameters

During the study period and in all streams, water temperature, conductivity (WTW LF 330) and pH (Jenway 3310) were measured 4–7 times. From each stream, 1 L of stream water was collected (4–7 times) in acid washed glass bottles. Water was filtered through glass fiber filters in the field (Millipore APFF) and transported in ice chests to the laboratory where subsamples for nitrate and soluble reactive phosphorus (SRP) determination were frozen. An unfiltered subsample was promptly analyzed for ammonia. Nitrate and ammonia concentrations were determined by ion chromatography (Dionex DX-120). SRP concentration

Table 1. Geographic characteristics of reference and nutrient enriched streams during the study period.

Stream pair	Stream	Altitude (m a.s.l.)	Latitude (N)	Longitude (W)	Stream order*	Distance to source (km)	Cat. area (km^2)	Geology	Dominant substrate type (cm)
Reference streams									
1	R1	620	40º 4' 44.4''	8º 12' 9.8''	2	1	0.8	schist	6–20
2	R2	520	40º 5' 20.8''	8º 12' 5.6''	3	2	2.9	schist	6–20
3	R3	570	40º 31' 59.4''	8º 13' 52.9''	3	2	2.4	granite	2–6
4	R4	805	40º 35' 43.7''	8º 10' 30''	3	1	0.8	schist	6–20
5	R5	255	40º 31' 28.8''	8º 11' 7.6''	4	4	3.2	schist	>40
Nutrient enriched streams									
1	N1	210	40º 32' 1.1''	8º 9' 14.8''	4	8	14.2	granite	0.2–2
2	N2	215	40º 32' 5.4''	8º 9' 30.8''	4	4	5.6	granite	0.2–2
3	N3	430	40º 34' 3.7''	8º 9' 2.7''	2	1	2.0	granite	0.2–2
4	N4	455	40º 34' 11.8''	8º 9' 3.6''	3	2	0.5	granite	>40
5	N5	245	40º 32' 27.6''	8º 9' 17.8''	5	13	43.1	granite	0.2–-2

*1:25 000 military maps (Strahler system)

Table 2. Physico-chemical characteristics (mean±1SD) of the water in reference and nutrient enriched streams during the study period (*n*=4–5).

Stream pair	Stream	Temperature (ºC)	Conductivity ($\mu S\ cm^{-1}$)	pH	Alkalinity ($mgCaCO_3\ L^{-1}$)	NO_3-N ($\mu g\ L^{-1}$)	NH_4-N ($\mu g\ L^{-1}$)	SRP ($\mu g\ L^{-1}$)
Reference streams								
1	R1	9.4 ± 0.7	24.8 ± 0.2	6.5 ± 0.1	3.8 ± 0.1	71.6 ± 5.1	<10–117	6.3 ± 0.4
2	R2	10.1 ± 0.9	30.2 ± 1.3	6.5 ± 0.2	5.1 ± 0.9	75.5 ± 8.1	<10–458	4.8 ± 0.5
3	R3	10.3 ± 0.7	27.0 ± 1.1	5.8 ± 0.1	1.8 ± 0.3	114.2 ± 36.0	<10	4.0 ± 0.4
4	R4	9.3 ± 0.6	24.8 ± 0.5	5.8 ± 0.0	0.7 ± 0.5	483.3 ± 59.1	<10	2.8 ± 0.4
5	R5	10.4 ± 0.8	27.0 ± 0.7	6.4 ± 0.1	3.8 ± 0.5	41.7 ± 8.4	<10	16.3 ± 2.1
Nutrient enriched streams								
1	N1	10.7 ± 0.6	53.6 ± 0.9	6.4 ± 0.1	4.9 ± 0.3	1126.7 ± 65.4	<10–13	29.1 ± 1.7
2	N2	10.9 ± 0.6	31.2 ± 1.0	6.4 ± 0.1	4.3 ± 0.6	216.2 ± 65.2	<10	25.0 ± 1.5
3	N3	10.8 ± 0.7	82.3 ± 5.8	6.7 ± 0.1	6.2 ± 0.8	2995.4 ± 332.7	<10	28.0 ± 3.2
4	N4	10.5 ± 0.5	59.4 ± 2.5	6.5 ± 0.2	5.5 ± 0.8	2153.0 ± 170.1	<10–209	26.2 ± 8.6
5	N5	11.0 ± 0.7	56.0 ± 2.4	6.6 ± 0.1	7.3 ± 0.2	1339.9 ± 203.3	120.3 ± 36.8	56.4 ± 21.7

was determined by the ascorbic acid method (APHA, 1995). Alkalinity was also determined in 3 occasions, by titration to an end pH of 4.5 (APHA, 1995).

Litter bags and decomposition

Alder (*Alnus glutinosa* (L.) GAERTNER) and oak (*Quercus robur* L.) leaves were collected from the same group of trees just after abscission in autumn 2002. Both leaf species were air-dried and stored dry until needed. On November 24, 2002, batches of 4.7–5.0 g of alder and oak leaves were weighed, rehydrated, and allocated into tetrahedral shaped fine mesh (FM; 10 x 15 cm, 0.5 mm mesh) and coarse mesh (CM; 10 x 15 cm, 10 mm mesh) bags and allocated in the streams in the following day. Since oak leaves decompose slowly they were lost in a flood in early January 2003. On January 26 and 27, 2003, new batches of 4.6–5.2 g of oak leaves were allocated in the same streams.

Groups of 2 bags (1 FM + 1 CM) were tied by nylon lines to iron rebars anchored to the stream bed. Alder leaves were retrieved after 7, 13, 20, 26 and 44 days in water in streams R1 and N1 whereas oak leaves were retrieved after 14, 26, 43, 57 and 74 days (4–6 replicates of each bag type, each time). In the other streams alder leaves were retrieved after 22 (N2 and N5) or 26 days (N3 and N4) and oak leaves after 42 (N2 and N5) or 57 (N3 and N4) days (6 replicates of each bag type). Sampling dates for bags in streams R2, N2 to R5, N5 were chosen to achieve approximately 50 % mass loss (T50) based on the decay rate in coarse mesh bags in stream R1. Extra sets of 4–6 leaf batches were prepared to calculate initial air dry mass to ash free dry mass (AFDM) conversion factors.

After retrieval, bags were placed in individual zip lock bags and transported in ice chests to the laboratory. Bags from streams R1 and N1 were processed within 24 h after retrieval while bags from streams R2, N2 to R5, N5 were frozen. In the laboratory, leaf remains from bags incubated in streams R1 and N1 were rinsed with distilled water onto a 0.5 mm mesh sieve to remove sediments and invertebrates, and 2 sets of 5 leaf disks (from CM bags) were cut out with a cork borer (12 mm diameter; see below). The remaining material was oven dry at 105 ºC for 24 h and weighed to calculate oven dry mass. A subsample was then ashed at 550 ºC for 4 h and reweighed to calculate % ash and AFDM. After defreeze, leaf remains from bags from streams R2, N2 to R5, N5 were rinsed with tap water onto a 0.5 mm mesh sieve to remove sediments and invertebrate, oven dry at 105 ºC for 24 h, weighed, ashed at 550 ºC for 4 h and reweighed to calculate AFDM remaining.

Fungal biomass

One set of leaf disks cut from leaves enclosed in coarse mesh bags incubated in streams R1 and N1 was used for ergosterol determination, as a measure of fungal biomass (GESSNER & SCHMITT, 1996; GESSNER, 2005). Leaf disks were frozen and later freeze-dried just before extraction. Immediately after dried, leaf disks were place in tightly closed tubes with 10 mL of KOH/methanol. Ergosterol was extracted in a water bath (80 ºC) for 30 min., with stirring. The extract was then purified by solid-phase extraction (Waters Sep-Pak Vac RC tC_{18} cartridges) as described by GESSNER (2005). Ergosterol was quantified by high performance liquid chromatography (HPLC) by measuring absorbance at 282 nm. The HPLC system (Dionex DX-120) was equipped with the reverse phase C_{18} column (Brownlee SPHERI-5RP-18, Applied Biosystems) maintained at 33 ºC. The mobile phase was 100 % methanol and the flow rate was set at 1.5 mL min^{-1}. Ergosterol was converted into fungal biomass with a conversion factor of 5.5 µg ergosterol mg^{-1} fungi dry mass (GESSNER & CHAUVET, 1993). Results were expressed as mg fungal biomass g^{-1} AFDM.

Aquatic hyphomycete sporulation

The 2nd set of leaf disks was used to induce sporulation by aquatic hyphomycetes (BÄRLOCHER, 2005). Disks were incubated in 100 mL Erlenmeyer flasks with 25 mL of filtered stream water (glass fiber filter, Millipore APFF), on an orbital shaker (100 rpm) for 48 h at 15 ºC. The conidia suspensions were decanted in 50 mL centrifuge tubes, flasks rinsed twice, and conidia fixed with 2 mL of 37 % formalin to be later counted and identified. When preparing slides for conidia identification, 100 µL of Triton X-100 solution (0.5 %) were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot of the suspension was filtered (Millipore SMWP, 5 µm pore size). Filters were stained with cotton blue in lactic acid (0.05 %), and spores were identified and counted with a compound microscope at 200x. Leaf disks were used to calculate remaining AFDM (as above). Sporulation rates were expressed as number of conidia released mg^{-1} AFDM day^{-1}.

Macroinvertebrates from litter bags

Invertebrates retained on 0.5 mm mesh sieve were preserved in 70 % ethanol and later identified to genus or species, except for Oligochaeta (family), Hidracarina and Ostracoda (presence) and some Diptera (subfamily or tribe), and classified into 2 groups: shredders and non-shredders (MERRIT & CUMMINS, 1996; TACHET *et al.*, 2000). Biomass of shredders was

determined by measuring the total length of each individual to the nearest 0.5 mm, and estimating biomass using body length-dry mass relationships (MEYER, 1989; BURGHERR & MEYER, 1997; BENKE *et al.*, 1999).

Benthic samples

On December 2 and 4, 2002 (during alder incubation) and on January 31 and February 1, 2003 (during oak incubation) benthic samples were collected from all streams, using a kick net (0.3 x 0.3 m opening and 0.5 mm mesh size). Samples were composed of 6 kicks (along 1 m for 30 sec. each) taken in the most representative habitats within the study reach. Samples were transported to the laboratory in ice chests and stored with 4 % formalin. After sorting, invertebrates were preserved in 70 % ethanol for later counting and identification as described above.

DATA ANALYSIS

Decomposition rates, *k*, were calculated by linear regression of ln transformed data (negative exponential model $M_t = M_o \cdot e^{-kt}$, where M_o is the initial mass, M_t is the remaining mass at time t, and *k* is the decomposition rate). For streams R1 and N1, slopes on ln-transformed data were compared using 3-way ANCOVA (stream and mesh type as categorical variables and time as continuous variable) followed by Tukey's test (ZAR, 1999). Overall comparison of decomposition rates (k d^{-1}) at T50 between reference and nutrient enriched streams was done by paired t test. Comparison of AFDM remaining (arcsine square root transformed) at T50 between reference and nutrient enriched streams for each stream pair was made by t test. Relationships between decomposition rates and nutrient concentrations in stream water were assessed by linear regression.

Comparisons of fungal biomass and sporulation rates (log(x+1) transformed) in coarse mesh bags between streams R1 and N1 were made by 2-way ANOVA with stream type and time as categorical variables. Comparisons of total number of species of aquatic hyphomycetes (log(x+1) transformed) in coarse mesh bags at each sampling date between streams R1 and N1 were made by paired t test.

Abundance and richness of total invertebrates and shredders (log(x+1) transformed) in coarse mesh bags at T50 were compared between reference and nutrient enriched streams within each stream pair by Mann-Whitney rank test (since it was not possible to achieve normality in data distribution) and for all stream pairs by paired t test for means. Relationships between those 4 litter bag invertebrate parameters and (a) NO_3-N and (b) SRP concentration in

stream water and (c) *k* values were assessed by linear regression. Abundance and richness of total invertebrates and biomass of shredders in coarse mesh bags were compared between streams R1 and N1 by 2-way ANOVA with stream type and time as categorical variables.

For benthic samples, two biotic indices (IBMWP and IASPT; ALBA-TERCEDOR, 1996; JÁIMEZ-CUÉLLAR *et. al*., 2004), 4 diversity indices (Shannon's diversity index (H'), Simpson's diversity index (D), Pielou's equitability index (E) and Berger-Parker's dominance index (B); WASHINGTON, 1984) and 45 metrics (BARBOUR *et al.*, 1999) were calculated. As there were 2 sampling dates, a average value was considered for the statistical analysis. Indices and metrics were compared between reference and nutrient enriched streams considering all stream pairs together, using paired t test. Relationships between indices and metrics and (a) NO_3-N and (b) SRP concentration in stream water and (c) *k* rates were assessed by Spearman rank correlations. Bonferroni adjustment was not performed. Correspondence analysis (CA) was performed on log(x+1) transformed benthic macroinvertebrate abundances (CANOCO 4.5; TER BRAAK & SMILAUER, 1998) and correlation between axis coordinates and NO_3-N and SRP concentration in stream water was assessed (Spearman rank correlation). Statistical analyses were performed with STATISTICA 6 unless otherwise indicated.

RESULTS

Decomposition

In streams R1–N1 both alder and oak leaves, in coarse mesh bags, decomposed significantly faster in the nutrient enriched than in the reference stream (ANCOVA, $p<0.001$; **Fig. 1**). For leaves in fine mesh bags the differences between nutrient enriched and reference streams were not significant (ANCOVA, $p>0.050$).

When the decay rates in the 10 streams was pair wise analyzed, there was a tendency for alder leaves, in both coarse and fine mesh bags, to decompose faster in the nutrient enriched than in the reference streams (**Fig. 2**). However, the difference between reference and nutrient enriched streams was significant only for 3 (CM bags) and 2 (FM bags) out of 5 stream pairs (t test, $p<0.003$; **Fig. 2**), which resulted in an overall non-significant difference in decomposition rates between stream types (paired t test, $p=0.080$ for CM bags and $p=0.114$ for FM bags; **Table 3**). Oak leaves decomposed faster in all nutrient enriched streams than in reference streams (**Fig. 2**), which resulted in an overall significantly higher decomposition rates in the nutrient enriched than in reference streams, for both coarse and fine mesh bags (paired t test, $p=0.019$ for CM bags and $p=0.030$ for FM bags; **Table 3**).

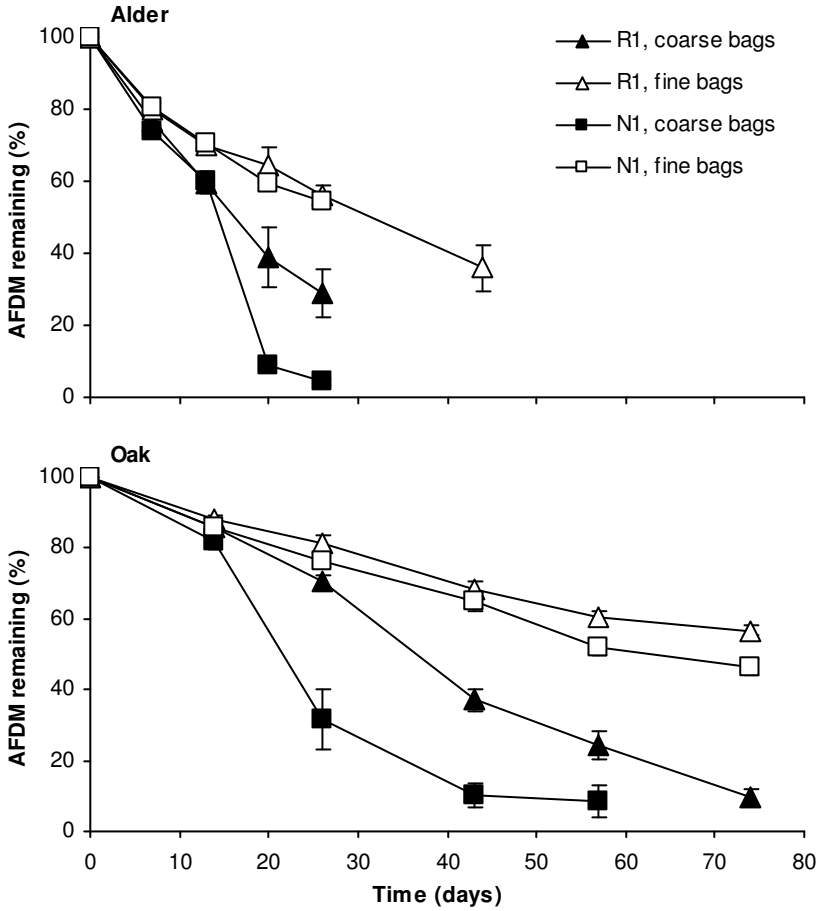

Figure 1. Remaining mass (mean±1SE) of alder and oak leaves in coarse and fine mesh bags incubated in reference (R1) and nutrient enriched (N1) streams.

Decomposition rates of both alder and oak leaves were related with the nitrogen and phosphorus concentrations in stream water. The increase in NO_3-N concentration in water up to approx. 1.5 mg L^{-1} led to an increase in the decomposition rates of both alder (up to k=0.147 d^{-1}) and oak (up to k=0.065 d^{-1}) leaves in coarse mesh bags (linear regression, $p<0.001$ and R^2=0.97 for alder and $p=0.009$ and R^2=0.70 for oak). When NO_3-N concentration in water increased beyond 1.5 mg L^{-1}, decomposition rates of both leaf species in coarse mesh bags decreased (**Fig. 3**). The relationship between decay rates and nitrogen was not significant for fine mesh bags (linear regression, $p>0.055$; **Fig. 3**).

Decomposition of alder and oak leaves was also related with phosphorus concentration in water. The increase in SRP concentration in water up to approx. 60 µg L^{-1} was accompanied by an increase of the decomposition rates of both leaf species in coarse mesh bags (linear

regression, p=0.005 and R^2=0.65 for alder and p=0.012 and R^2=0.57 for oak) and alder leaves in fine mesh bags (linear regression, p=0.006 and R^2=0.63) (**Fig. 4**).

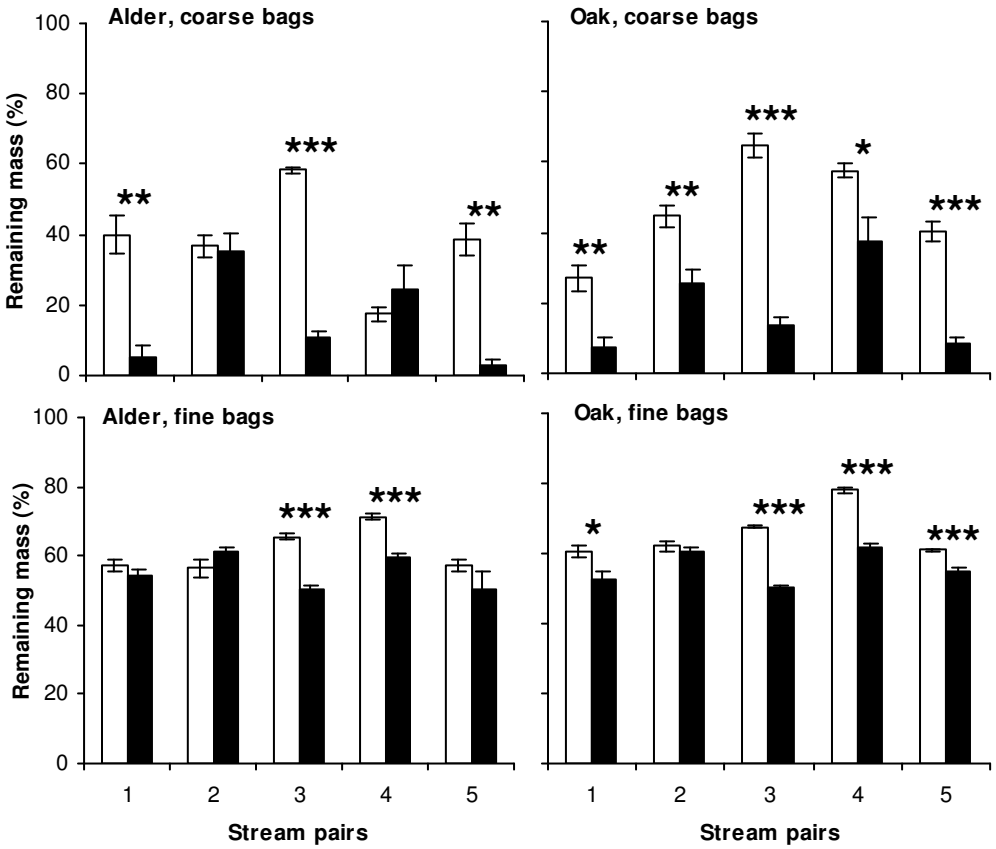

Figure 2. Remaining mass (mean±1SE) of alder and oak leaves in coarse and fine mesh bags incubated in 5 reference and 5 nutrient enriched streams. T test: *, p<0.050; **, p<0.010; ***, p<0.001.

Table 3. Decomposition rates, *k* day^{-1}, (95 % CL) of alder and oak leaves in coarse and fine mesh bags incubated in 5 reference (R) and 5 nutrient enriched (N) streams.

	Alder		Oak	
Stream	**Coarse mesh**	**Fine mesh**	**Coarse mesh**	**Fine mesh**
R1	0.036 (0.005)	0.022 (0.001)	0.024 (0.002)	0.009 (0.001)
R2	0.039 (0.003)	0.022 (0.002)	0.014 (0.001)	0.008 (<0.001)
R3	0.021 (0.001)	0.016 (0.001)	0.008 (0.001)	0.007 (<0.001)
R4	0.069 (0.005)	0.013 (<0.001)	0.010 (0.001)	0.004 (<0.001)
R5	0.037 (0.004)	0.022 (0.001)	0.016 (0.001)	0.009 (<0.001)
N1	0.133 (0.022)	0.024 (0.001)	0.065 (0.008)	0.011 (0.001)
N2	0.042 (0.006)	0.019 (0.001)	0.025 (0.003)	0.009 (<0.001)
N3	0.088 (0.006)	0.026 (0.001)	0.036 (0.003)	0.012 (<0.001)
N4	0.065 (0.014)	0.020 (0.001)	0.019 (0.004)	0.009 (<0.001)
N5	0.147 (0.022)	0.031 (0.004)	0.051 (0.011)	0.010 (<0.001)
p (paired t test)	0.080	0.114	**0.019**	**0.030**

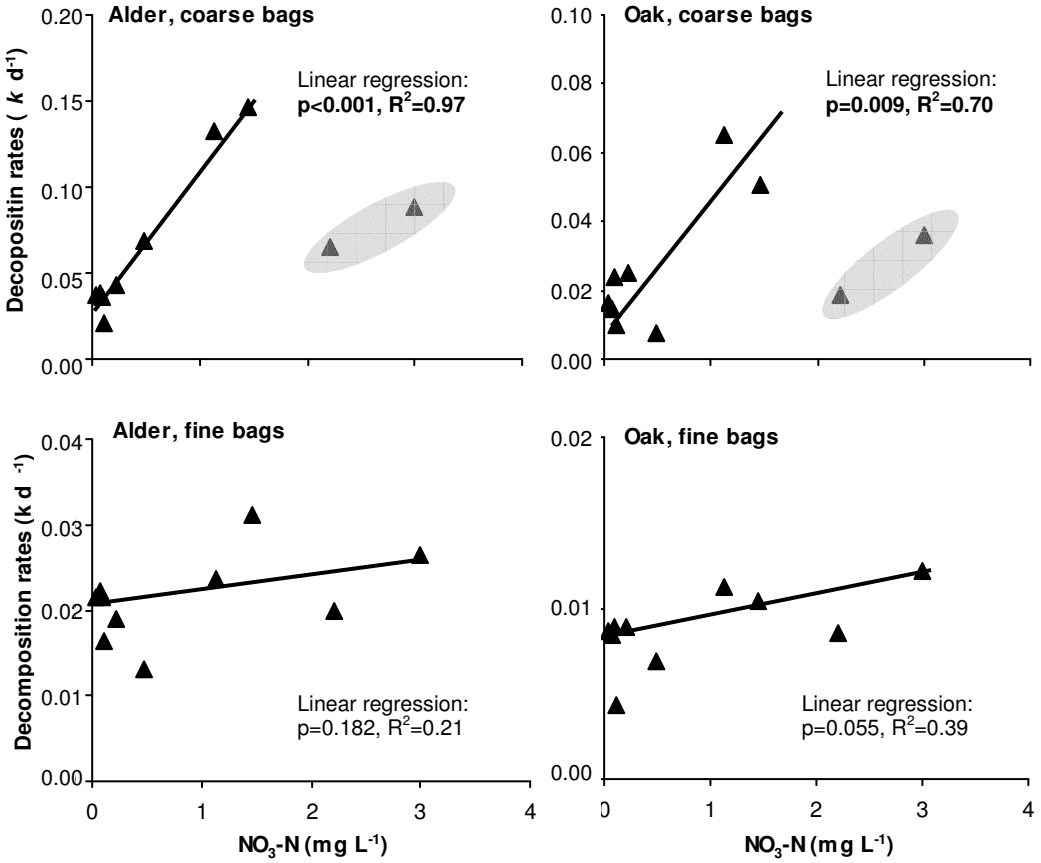

Figure 3. Relationships between decomposition rates (k d^{-1}) of alder and oak leaves in coarse and fine mesh bags and NO_3-N concentrations in stream water.

Fungal biomass

At day 0, fungal biomass was 5.4 mg g^{-1} AFDM for oak and 10.8 mg g^{-1} AFDM for alder, reveling some fungal colonization of leaves at the beginning of the experiment. Dynamics of fungal biomass in alder leaves was similar in both streams with a rapid increase to a maximum of 106 mg g^{-1} AFDM by day 13 in the nutrient enriched stream and a maximum of 112 mg g^{-1} AFDM one week later in the reference stream (**Fig. 5**). For this leaf species, there were no significant differences in fungal biomass between streams (2-way ANOVA, p=0.721). Fungal biomass associated with oak leaves increased over time until the last sampling date (day 57 with 112 mg g^{-1} AFDM) in the nutrient enriched stream while in the reference stream it peaked by day 57 with 86 mg g^{-1} AFDM (**Fig. 5**). Fungal biomass associated with oak leaves was significantly higher in the nutrient enriched than in the reference stream (2-way ANOVA, p<0.001).

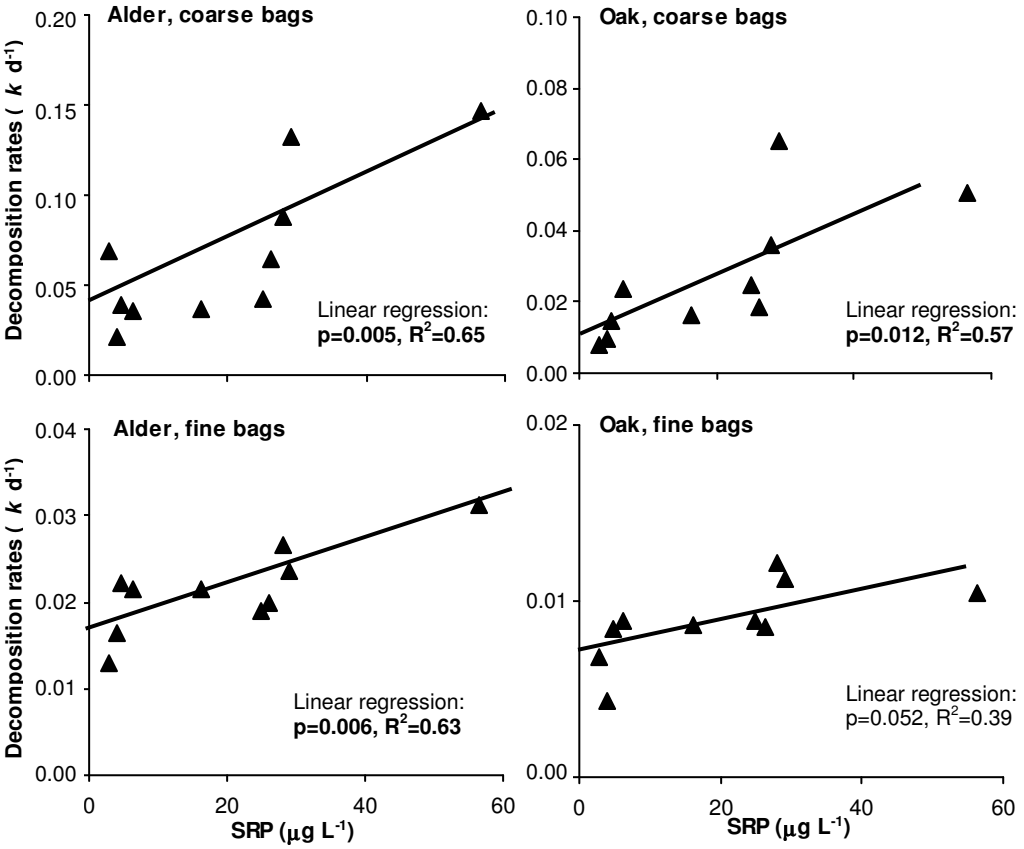

Figure 4. Relationships between decomposition rates (k d^{-1}) of alder and oak leaves in coarse and fine mesh bags and SRP concentrations in stream water.

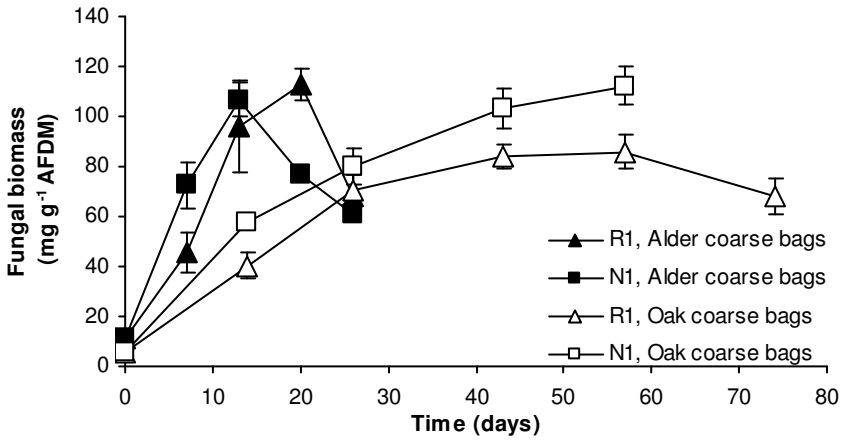

Figure 5. Fungal biomass (mean±1SE) associated with alder and oak leaves incubated in reference (R1) and nutrient enriched (N1) streams.

Aquatic hyphomycete sporulation

Sporulation rate of aquatic hyphomycetes associated with alder leaves peaked first and at higher level in the nutrient enriched than in the reference stream, however, there were no overall differences between streams (2-way ANOVA, p=0.300; **Fig. 6**). Sporulation rate in oak leaves peaked by day 26 (**Fig. 6**) and was higher in the nutrient enriched than in the reference stream (2-way ANOVA, p=0.010).

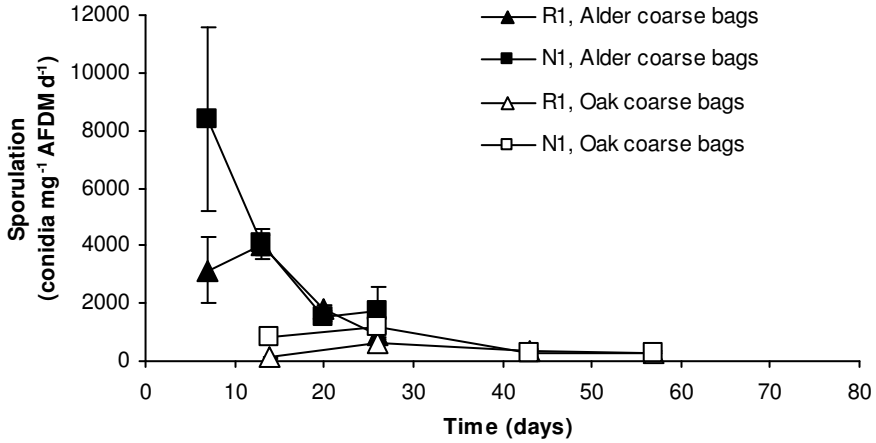

Figure 6. Fungal sporulation rate (mean±1SE) of aquatic hyphomycetes associated with alder and oak leaves incubated in reference (R1) and nutrient enriched (N1) streams.

Aquatic hyphomycetes community structure

The number of species of aquatic hyphomycetes on decomposing leaves peaked earlier in the nutrient enriched than in the reference stream (**Fig 7**). For both alder and oak leaves there was no significant difference in species richness between the nutrient enriched and the reference stream (paired t test, p=0.219 for alder and 0.379 for oak).

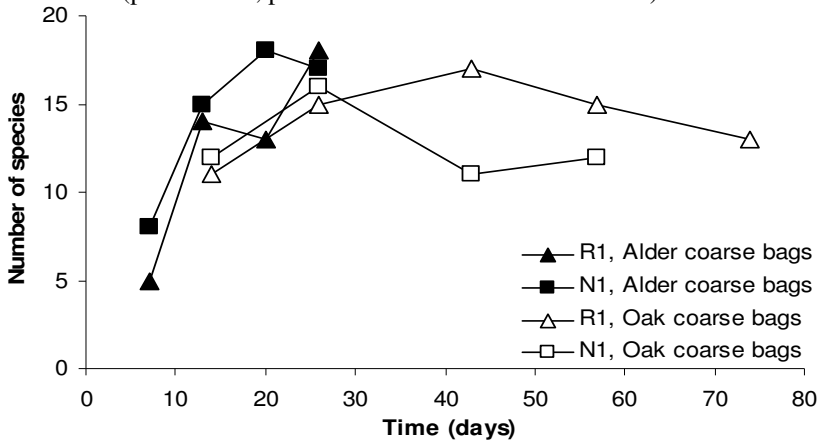

Figure 7. Number of species of aquatic hyphomycetes in alder and oak leaves incubated in reference (R1) and nutrient enriched (N1) streams.

The total number of aquatic hyphomycetes species identified ranged from 18 (alder in R1 and oak in N1) to 23 (alder in N1; **Table 4**). *Tetrachaetum elegans* was the dominant species in alder bags at both streams (31–32 % of total spore production), followed by *Crucella subtilis* (28 %; alder in R1) and *Flagellospora curvula* (28 %; alder in N1). *Tricladium chaetocladium* was the dominant species in oak bags at both streams (34 % in R1 and 45 % in N1). At the sporulation peak, the same species were dominant (**Table 4**). Among the abundant species (>5 % of spores in at least one substrate), *Anguillospora filiformis*, *Clavatospora longibrachiata* and *Tricladium chaetocladium* had higher relative abundances in the nutrient enriched than in the reference stream while the opposite was observed for *Stenocladiella neglecta*. *Crucella subtilis* was the only abundant species appearing only in the reference stream (**Table 4**).

Macroinvertebrates

Fifty-eight taxa of invertebrates (17–26 families per substrate and stream type) were recovered from litter bags. Shredders were represented by 21 taxa (6–8 families; mainly Plecoptera and Trichoptera; **Table 5**). Up to 27 taxa were identified in a single stream (alder in N1). The most common taxa were *Allogamus* sp., Orthocladiinae and Chironomini and the most common shredders were *Allogamus* sp., *Protonemura* sp., *Stenophylax* sp. and *Sericostoma* sp.

There were no overall significant differences between reference and nutrient enriched streams for alder bags in terms of abundance and richness of total invertebrates and shredders g^{-1} AFDM (paired t test, $p>0.179$; **Fig. 8a–d**). Only for stream pair 3 were invertebrate numbers significantly higher in the nutrient enriched than in the reference stream (Mann-Whitney rank test, $p<0.026$; **Fig. 8a–d**). Oak leaves in nutrient enriched streams had significantly higher abundance and richness of total invertebrates g^{-1} AFDM than reference streams (paired t test, $p=0.032$ and 0.016, respectively; **Fig. 8e–f**). No significant differences between reference and nutrient enriched streams were found for abundance and richness of shredders g^{-1} AFDM (paired t test, $p>0.068$; **Fig. 8g–h**), for which only stream pair 3 had numbers significantly higher in the enriched than in the reference stream (Mann-Whitney rank test, $p=0.002$; **Fig. 8g–h**). Abundance and richness of total invertebrates and abundance of shredders g^{-1} AFDM in oak bags were positively related with NO_3-N concentration in stream water (linear regression, $p=0.018$ and $R^2=0.53$, $p=0.019$ and $R^2=0.52$ and $p=0.020$ and $R^2=0.51$, respectively; **Table 6**).

Table 4. Relative abundances (%; means over all sampling dates) of aquatic hyphomycete conidia released from alder and oak leaves incubated in the reference (R) and in the nutrient enriched (N) stream. Between brackets, percent contribution at peak sporulation time. +, contribution <0.1 %.

	Alder		Oak	
Aquatic hyphomycetes species	**R**	**N**	**R**	**N**
Alatospora acuminata INGOLD	0.7 (1.1)	1.2	6.1 (0.5)	1.6 (0.2)
Alatospora flagellata (GÖNCZÖL) MARVANOVÁ			0.1	
Alatospora pulchella MARVANOVÁ	0.4 (0.1)		2.2 (0.1)	0.5
Anguillospora crassa INGOLD				
Anguillospora filiformis GREATHEAD	0.4	1.4 (1.8)	13.5 (1.2)	16.4 (4.6)
Anguillospora longissima (SACC & SYD.) INGOLD				
Anguillospora cf. *furtiva* WEBSTER & DESCALS		+		
Articulospora tetracladia INGOLD	0.3 (0.5)	0.6	1.3 (1.6)	2.6 (1.7)
Clavariopsis aquatica DE WILD.	1.1 (0.1)	2.0 (0.6)	5.9 (3.4)	4.3 (6.5)
Clavatospora longibrachiata (INGOLD) MARVANOVÁ & S.NILSSON	10.5 (2.0)	15.0 (0.2)	8.6 (0.7)	11.2 (3.7)
Crucella subtilis MARVANOVÁ & SUBERKROPP	27.9 (33.4)		3.2 (5.5)	
Culicidospora aquatica R.H. PETERSEN	0.1 (0.1)		0.1	
Flagellospora curvula INGOLD	13.6 (24.3)	28.1 (29.5)	2.6 (5.9)	2.3 (5.2)
Goniopila monticola (DYKO) MARVANOVÁ & DESCALS	0.6	0.4	5.2 (0.1)	
Heliscella stellata (INGOLD & COX) MARVANOVÁ & NILSSON	0.1	0.9	0.1	0.2 (0.1)
Heliscus lugdunensis SACC. & THÉRRY		0.1	+ (0.1)	0.1
Lemonniera aquatica DE WILD.		+		0.2 (0.1)
Lunulospora curvula INGOLD		0.4 (0.2)		
Stenocladiella neglecta (MARVANOVÁ & DESCALS) MARVANOVÁ & DESCALS	9.3 (2.0)	4.2	4.0 (1.7)	1.7 (0.6)
Taeniospora gracilis var. *enecta* MARVANOVÁ & IQBAL	0.5 (0.9)	0.2	0.1 (1.1)	0.3 (0.3)
Tetrachaetum elegans INGOLD	31.5 (31.9)	32.1 (62.3)	12.7 (33.0)	13.1 (17.3)
Tricladium chaetocladium INGOLD	0.4 (0.3)	11.7 (5.3)	33.6 (45.9)	44.7 (58.8)
Tricladium splendens INGOLD			+	0.1 (0.1)
Triscelophorus acuminatus NAWAWI	0.2	0.7		0.1 (0.1)
Triscelophorus monosporus INGOLD		+		
Tumularia aquatica (INGOLD) DESCALS & MARVANOVÁ		+		
Varicosporium elodeae KEGEL		0.1		
Unidentified tetraradiate	0.1 (0.1)	0.1	0.1 (0.1)	0.2 (0.1)
small sigmoid (<60 μm)	2.3 (3.1)	0.5	0.3	0.3 (0.6)
large sigmoid (>120 μm)		+ (0.2)		
Total no. species	18	23	20	18
No. species at sporulation peak time	14	8	15	16
Days in water at sporulation peak time	13	7	26	26

During decomposition, abundance and richness of total invertebrates and biomass of shredders colonizing both alder and oak leaves increased over time in the reference stream (R1), while in the nutrient enriched stream (N1) a peak in numbers was achieved before the end of the experiment. There was also a tendency for peak in numbers to be higher in the nutrient enriched than in the reference stream (**Fig. 9**), however, no significant differences were found between streams (2-way ANOVA, $p>0.109$).

Table 5. Macroinvertebrate families recovered from decomposing alder and oak leaves incubated in 5 reference and 5 nutrient enriched streams. Values indicate the number of streams (maximum=5) in which taxa occurred.

	Alder		Oak	
Litter bag macroinvertebrates	**Reference**	**Nutrient**	**Reference**	**Nutrient**
Baetidae	3	1	2	3
Caenidae		1		
Ephemerellidae		1		4
Ephemeridae				1
Leptophlebiidae*			1	
Leuctridae*	1	1		
Nemouridae*	4	1	3	2
Chloroperlidae	1		2	
Calamoceratidae*		1	1	2
Hydropsychidae	2	4	1	3
Lepidostomatidae*		3	1	3
Leptoceridae*	1			
Limnephilidae*	4	3	3	5
Philopotamidae	1			
Policentropodidae			1	1
Psychomyiidae		1		
Rhyacophilidae	2	2	2	3
Sericostomatidae*		2	1	2
Dytiscidae		1		
Scirtidae*	2		1	
Calopterygidae		1		1
Nepidae				1
Hydrobiidae		1		1
Physidae		1		
Acari			1	1
Ostracoda	1	1		
Aselidae*			1	
Athericidae	2	3	1	3
Chironomidae	4	4	2	5
Dixidae			1	1
Empididae	1	3	1	1
Simuliidae	2	1	1	1
Psychodidae		1		
Tipulidae*	2	1		1
Lumbriculidae		1		1
Naididae	1	1		1
Erpobdellidae		1		2
Total no. families	17	26	19	24
No. shredder families	6	7	8	6

*, shredders

From benthic samples, a total of 154 macroinvertebrate taxa was identified in the 10 streams, varying between 38 and 51 taxa in reference, and between 39 and 51 in nutrient enriched streams. Twelve families, being the most abundant Sericostomatidae, Nemouridae,

Leuctridae and Leptophlebiidae, represented shredders. The taxa richness of shredders varied between 10 and 14 in reference, and between 7 and 9 in nutrient enriched streams. Reference streams had significantly higher abundance and richness of shredders than nutrient enriched streams (paired t test, p=0.017 and <0.001, respectively) (**Table 7**).

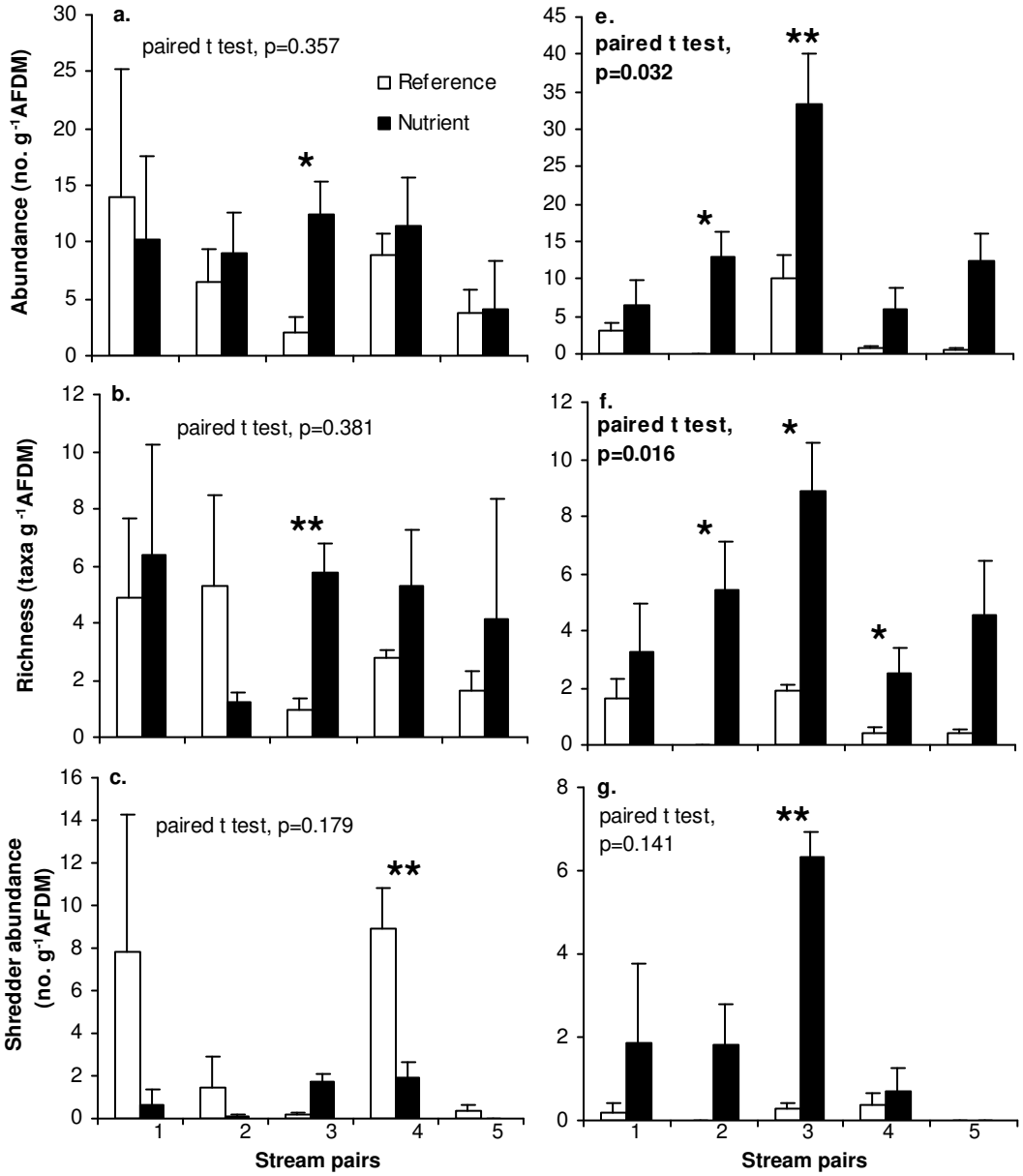

Figure 8. Abundance and richness of total invertebrates and shredders g^{-1} AFDM (mean±1SE) associated with alder and oak leaves incubated in 5 reference and 5 nutrient enriched streams. Mann-Whitney rank test; *, p<0.050; **, p<0.010.

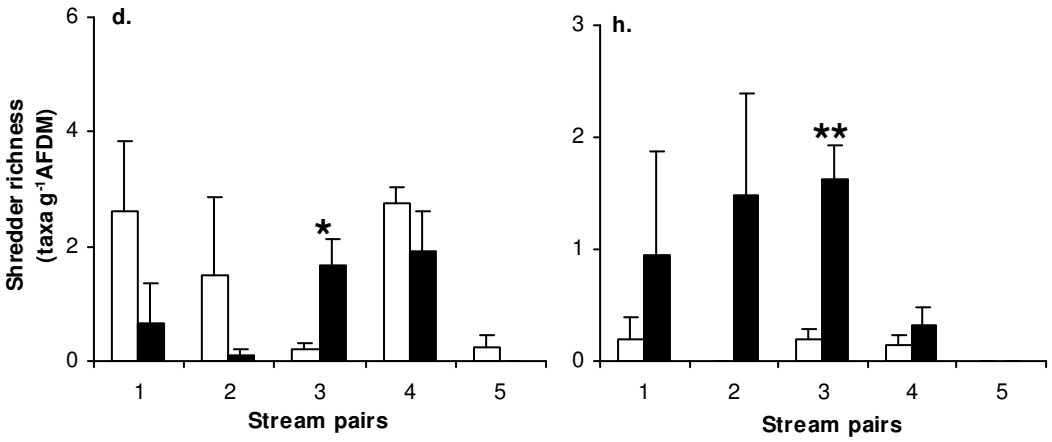

Figure 8. Continued

Table 6. Linear regression (p values) between abundance and richness of total invertebrates and shredders colonizing alder and oak leaves (no. g^{-1} AFDM) incubated in all 10 streams and NO_3-N and SRP concentration in stream water and respective decomposition rates.

	Total invertebrates		Shredders	
	Abundance	**Richness**	**Abundance**	**Richness**
Alder				
NO_3-N in water	0.226	0.793	0.646	0.678
SRP in water	0.919	0.448	0.129	0.159
Decomposition rate	0.780	0.107	0.615	0.645
Oak				
NO_3-N in water	**0.018**	**0.019**	**0.020**	0.174
SRP in water	0.193	0.064	0.512	0.493
Decomposition rate	0.331	0.130	0.345	0.288

Assessment of environmental quality

When stream ecological integrity was assessed by the absolute decomposition rate (k d^{-1}) value of alder leaves incubated in coarse mesh bags, as suggested by GESSNER & CHAUVET (2002), 9 streams were classified as compromised or severely compromised while by using the decomposition rate value of alder leaves incubated in fine mesh bags only 2 streams (N3 and N5) were considered as compromised (**Table 8**). According to the same classification, the ratio coarse/fine mesh bags k value for both alder and oak leaves classified 9 streams as compromised or severely compromised (**Table 8**). Stream functional integrity was also assessed by the ratio of impaired/reference streams k value (GESSNER & CHAUVET, 2002). Decomposition of oak leaves in coarse mesh bags was more sensitive to nutrient enrichment than decomposition in fine mesh bags or alder leaves in both coarse or fine mesh bags (**Table 9**).

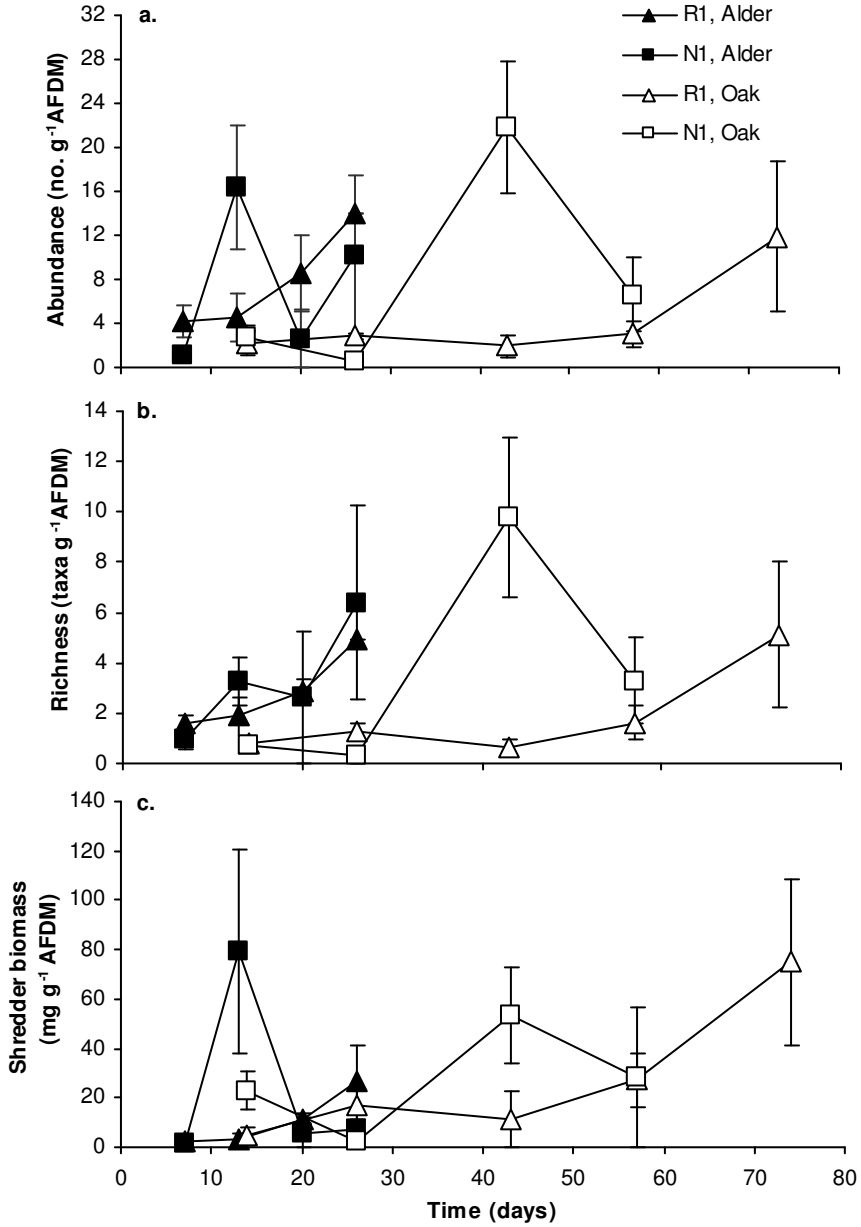

Figure 9. Abundance and richness of total invertebrates and biomass of shredders g^{-1} AFDM (mean±1SE) in alder and oak leaves incubated in reference (R1) and nutrient enriched (N1) streams.

The IBMWP, but not IASPT, biotic index values were significantly higher at reference than at nutrient enriched streams (paired t test, p=0.006; **Table 10**), although no stream was classified as polluted. Both IBMWP and IASPT were negatively correlated with nitrogen concentration in stream water (Spearman rank correlation, p=0.022 and 0.009, respectively;

Table 11). Nutrient enriched and reference streams were not significantly different in terms of diversity parameters (paired t test, p>0.913; **Table 11**).

Table 7. Abundance and richness of total invertebrates and shredders (mean number of both sampling occasions) in 5 reference (R) and 5 nutrient enriched (N) streams.

	Total invertebrates		Shredders	
Stream	**Abundance**	**Richness**	**Abundance**	**Richness**
R1	1164	51	351	14
R2	906	43	397	10
R3	866	42	171	13
R4	542	38	243	10
R5	521	50	153	12
N1	816	51	170	9
N2	582	44	38	7
N3	381	49	47	8
N4	435	39	10	5
N5	1203	45	80	8
p (paired t test)	0.607	0.777	**0.017**	**<0.001**

Table 8. Decomposition rates (k day^{-1}) of alder leaves in coarse mesh and fine mesh bags and ratios of alder and oak decomposition rates in coarse mesh bags to decomposition rates in fine mesh bags (k_c/k_f) in 5 reference (R) and 5 nutrient enriched (N) streams. Unshaded, no clear evidence of impact; light grey, compromised river functioning and dark grey, severely compromised river functioning (as defined by GESSNER & CHAUVET, 2002).

	Alder		Coarse/Fine	
Stream	**Coarse mesh**	**Fine mesh**	**Alder**	**Oak**
R1	0.036	0.022	1.65	2.65
R2	0.039	0.022	1.75	1.71
R3	0.021	0.016	1.27	1.12
R4	0.069	0.013	5.28	2.25
R5	0.037	0.022	1.71	1.87
N1	0.133	0.024	5.64	5.76
N2	0.042	0.019	2.23	2.82
N3	0.088	0.026	3.33	2.96
N4	0.065	0.020	3.25	2.20
N5	0.147	0.031	4.70	4.85

Table 9. Ratios of alder and oak decomposition rates in nutrient enriched streams to decomposition rates in reference streams (k_N/k_R) in coarse mesh and fine mesh bags. Unshaded, no clear evidence of impact; light grey, compromised river functioning and dark grey, severely compromised river functioning (as defined by GESSNER & CHAUVET, 2002).

	Alder		Oak	
Stream	**Coarse mesh**	**Fine mesh**	**Coarse mesh**	**Fine mesh**
N1/R1	3.73	1.09	2.75	1.27
N2/R2	1.09	0.86	1.74	1.06
N3/R3	4.26	1.62	4.67	1.76
N4/R4	0.94	1.53	1.92	1.97
N5/R5	3.98	1.45	3.13	1.21

Table 10. Values of the biotic indices IBMWP and IASPT in 5 reference (R) and 5 nutrient enriched (N) streams during the decomposition experiment. IBMWP is the sum of the tolerance scores of families (score=1, very tolerant to organic pollution to score=10, very sensitive to organic pollution) represented by 2 or more individuals. IASPT is the average score per taxon (=IBMWP/ number of IBMWP families). IBMWP>100, good water quality (class 1 out of 5, as defined by ALBA-TERCEDOR, 1996; JÁIMEZ-CUÉLLAR *et al.*, 2004).

	IBMWP	**IASPT**
R1	214	6.44
R2	150	7.66
R3	138	6.53
R4	134	6.64
R5	159	6.75
N1	179	6.61
N2	119	6.42
N3	128	5.56
N4	108	6.14
N5	116	5.40
p (paired t test)	**0.006**	0.096

Among the 45 metrics computed, only 14 discriminated reference from nutrient enriched streams: % EPT taxa, Baetidae/Total Ephemeroptera (# ind), number and % of Plecoptera taxa and individuals, number and % of Oligochaeta taxa, number and % of intolerant taxa and number and % of shredder taxa and individuals (paired t test, $p<0.042$; **Table 11**). From these 14 metrics, 10 and 12 were significantly correlated with NO_3-N and SRP concentration in water, respectively; 3 metrics were significantly correlated with alder *k* and 3 with oak *k* values (**Table 11**).

Table 11. Predicted and observed response of 2 biotic indices, 4 diversity indices and 45 metrics (applied to the benthic macroinvertebrate community) to nutrient enrichment of stream water. Paired t test for means was performed between reference (R) and nutrient enriched (N) streams, considering all stream pairs together: =, no significant difference between stream types ($p>0.050$); >, significant difference between stream types ($p<0.050$). For Spearman rank correlations: *, $p<0.050$; **, $p<0.010$; (+), positive correlation; (-), negative correlation.

	Reference vs. Nutrient: paired t test		**Metrics *vs.* nutrients in water: correlation**		**Metrics *vs. k* values: correlation**	
Indices and Metrics	**Predicted**	**Observed**	**NO_3-N**	**SRP**	**Alder**	**Oak**
IBMWP	R>N	**R>N**	* (–)			
IASPT	R>N	N=R	** (–)			
Shannon's diversity index, H'	R>N					
Simpson's diversity index, D	N>R					
Pielou's equitability index, E	R>N					
Berger-Parker's index, B	N>R					
Total no. inds	?	N=R				
Total no. taxa	R>N	N=R				
No. EPT taxa	R>N	N=R	** (–)			
% EPT taxa	R>N	**R>N**	** (–)	** (–)		
No. EPT inds	R>N	N=R				

Table 11. Continued

	Reference vs. Nutrient: paired t test		Metrics *vs.* nutrients in water: correlation		Metrics *vs. k* values: correlation	
Indices and Metrics	**Predicted**	**Observed**	**NO_3-N**	**SRP**	**Alder**	**Oak**
% EPT inds	R>N	N=R				
No. Ephemeroptera taxa	R>N	N=R				
% Ephem taxa	R>N	N=R				
No. Ephem inds	R>N	N=R				
% Ephem inds	R>N	N=R				
No. Baetidae inds	N>R	N=R				
Baetidae/Total Ephem (# inds)	N>R	**N>R**	* (+)	** (+)		* (+)
No. Plecoptera taxa	R>N	**R>N**	* (–)	** (–)		
% Plec taxa	R>N	**R>N**	* (–)	** (–)		
No. Plec inds	R>N	**R>N**	* (–)	** (–)		
% Plec inds	R>N	**R>N**	* (–)	** (–)		
No. Trichoptera taxa	R>N	N=R	* (–)			
% Trichopt taxa	R>N	N=R	** (–)	* (–)	* (–)	
No. Trichopt inds	R>N	N=R				
% Trichopt inds	R>N	N=R				
No. Hydropsychidae inds	N>R	N=R				
Hydrops/ Total Trichopt (# inds)	N>R	N=R				
No. Diptera taxa	R>N	N=R				* (+)
% Diptera taxa	R>N	N=R	** (+)			
No. Diptera inds	N>R	N=R				
% Diptera inds	N>R	N=R				
No. Chironomidae taxa	N>R	N=R				
% Chironomidae taxa	N>R	N=R				
No. Chironomidae inds	N>R	N=R				
% Chironomidae inds	N>R	N=R				
No. Tanytarsini inds	R>N	N=R				
% Tanytarsini inds	R>N	N=R				
No. Oligochaeta taxa	N>R	**N>R**		** (+)		
% Oligo taxa	N>R	**N>R**		* (+)		
No. Oligo inds	N>R	N=R				
% Oligo inds	N>R	N=R				
EPT/Chironomidae (# inds)	R>N	N=R				
No. intolerant taxa[1]	R>N	**R>N**	** (–)			
% intolerant taxa	R>N	**R>N**	** (–)	* (–)		
No. tolerant taxa[2]	N>R	N=R				
% tolerant taxa	N>R	N=R				
No. Shredder taxa	R>N	**R>N**	* (–)	* (–)	* (–)	
% Shredders taxa	R>N	**R>N**		* (–)	* (–)	* (–)
No. Shredders inds	R>N	**R>N**				
% Shredders inds	R>N	**R>N**	* (–)	* (–)		

EPT, Ephemeroptera+Plecoptera+Trichoptera
[1] taxa with scores of 7, 8 and 10 in the IBMWP index
[2] taxa with scores of 1, 2 and 3 in the IBMWP index

When all the benthic community was considered (CA), reference streams were discriminated from nutrient enriched streams in both sampling dates (**Fig. 10**). Streams appeared distributed along axis 1, which was significantly correlated with SRP concentration in water (Spearman rank correlation, p=0.001 and r=0.91 (during alder incubation) and p=0.007 and r=0.80 (during oak incubation)).

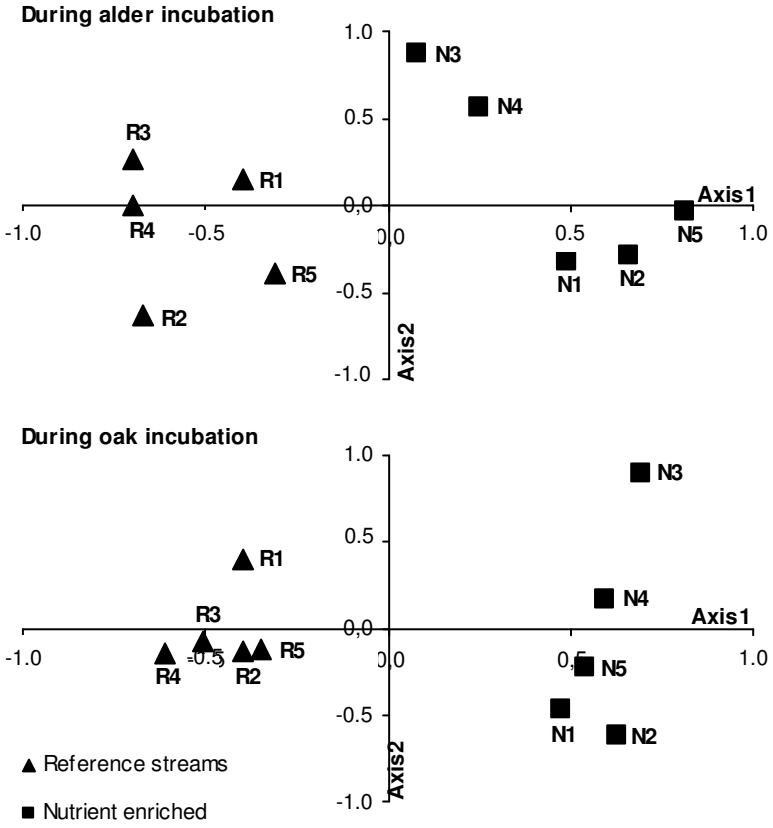

Figure 10. Ordination (CA) of 5 reference (R) and 5 nutrient enriched (N) streams along axis 1 and axis 2 according to their benthic macroinvertebrate community during alder and oak incubation. Axis 1 explaining 28.1 % (during alder incubation) and 26.4 % (during oak incubation) and axis 2 explaining 15.5 % (alder) and 16.8 % (oak) of the variability.

DISCUSSION

Previous studies reported stimulation of litter decomposition and associated microbial activity at relatively low levels of nutrients (DIN<300 μg L^{-1} and SRP<50 μg L^{-1}; GULIS & SUBERKROPP, 2003b; GULIS *et al.*, 2004). So, to be possible to detect a response of the aquatic communities and ecosystem-level processes to nutrient enrichment it is important to make comparisons with really oligotrophic reference streams. The reference streams in this study

had very low SRP (2.8–16.3 μg L^{-1}) and NO_3-N (41.7–483.3 μg L^{-1}) concentration that allowed detecting an increase in litter decomposition rates and associated microbial activity and invertebrate numbers in nutrient enriched streams.

In general, there was a tendency for higher decomposition rates in nutrient enriched than in reference streams (up to 4 and 5x in CM bags for alder and oak, respectively, and up to 2x in FM bags). The overall nutrient stimulation was, however, significant only for oak leaves, as expected since this is a low nutrient quality substrate where the microbial community is probably nutrient limited (STELZER *et al*., 2003; GULIS *et al*., 2004; FERREIRA *et al., in press*). Our results agree with other studies comparing decomposition rates of leaves in streams differing in nutrient content (SUBERKROPP, 1995; SUBERKROPP & CHAUVET, 1995; WEYERS & SUBERKROPP, 1996), or along a nutrient gradient (PASCOAL *et al.*, 2001, 2003; NIYOGI *et al.*, 2003). They are also consistent with results from nutrient manipulative experiments, in microcosms (e.g. SRIDHAR & BÄRLOCHER, 2000; ROSEMOND *et al.*, 2002), with diffusion substrates in streams (ROBINSON & GESSNER, 2000), in stream side channels (GRATTAN II & SUBERKROPP, 2001) and in entire streams (GULIS & SUBERKROPP, 2003b; FERREIRA *et al., in press*).

Decomposition rates of both alder and oak leaves in coarse mesh bags increased linearly with increasing N (up to 1.5 mg L^{-1}) and SRP concentration in water. Above 1.5 mg N L^{-1} mass loss slowed down that could result from an inhibitory effect of other pollutants associated with high nutrient concentration (e.g. pesticides). High nitrate concentrations (>30 mg L^{-1}) was previously reported to have an inhibitory effect on fungal activity (SRIDHAR & BÄRLOCHER, 1997), however, that was probably not the case here where maximum N concentrations were lower than the ones used by SRIDHAR & BÄRLOCHER (1997). A linear relationship between P concentration in water and breakdown rates was also found by SUBERKROPP & CHAUVET (1995) for yellow poplar leaves in Alabama (US) streams and NIYOGI *et al*. (2003) for tussock grass in New Zealand streams.

Decomposition rates in coarse mesh bags were stimulated to a greater extent than in fine mesh bags. It is not clear the reason for this difference between bag types in the response to N concentration in water, but it is plausible that the enrichment effect could have been amplified by invertebrate feeding since microbial colonization cause increase of feeding rates by detritus consumers, enhance growth and reproductive output (GRAÇA, 1993; GRAÇA *et al*., 2001) and potentially increase production.

Fungal biomass and sporulation rates of aquatic hyphomycetes were generally higher in the nutrient enriched than in the reference stream. This stimulation of fungal activity at high nutrient concentration is well documented both by laboratory (SRIDHAR & BÄRLOCHER, 1997; SUBERKROPP, 1998; GULIS & SUBERKROPP, 2003a) and stream (SUBERKROPP & CHAUVET, 1995; NIYOGI *et al*., 2003; GULIS & SUBERKROPP, 2003b; FERREIRA *et al., in press*) experiments. Oak litter, the low quality species, was more sensitive to nutrient enrichment than alder litter, which agree with previous reports by GULIS & SUBERKROPP (2003b) and FERREIRA *et al*. (*in press*). Also, sporulation rates were stimulated to a greater extent than biomass (average difference between reference and nutrient enriched stream: 1.6 *vs*. 1.1 (alder) and 2.7 *vs*. 1.2 (oak)), as demonstrated before (SUBERKROPP, 1998; GRATTAN II & SUBERKROPP, 2001).

The relationship between nutrients and species richness is not clear. We can predict an increase in the number of species of aquatic hyphomycetes with nutrient enrichment given that more nutrients may allow more species to be able of colonizing leaf substrates. This was observed by GULIS & SUBERKROPP (2003b) who found higher species richness on maple and rhododendron leaves in a nutrient enriched reach than in the reference one. On the other hand, more nutrients may give a high competitive advantage to some species and richness can decrease. Neither of these possibilities was observed in this study where no significant differences were found in species richness of aquatic hyphomycetes between streams. SRIDHAR & BÄRLOCHER (2000), in laboratory experiments, and FERREIRA *et al*. (*in press*), in a whole-stream enrichment experiment, also didn't found significant differences in species richness of aquatic hyphomycetes colonizing leaf litter at different nutrient concentrations. In this study, three common species (*Anguillospora filiformis*, *Clavatospora longibrachiata* and *Tricladium chaetocladium*) had their sporulation stimulated at high nutrient concentration while one species (*Stenocladiella* neglecta) was inhibited. This response of individual species to nutrient enrichment has also been notice in other studies (SRIDHAR & BÄRLOCHER, 2000; GULIS & SUBERKROPP, 2003b; FERREIRA *et al*, *in press*). *Crucella subtilis* was present only on the reference stream, although it is not possible to know if this was the result of differences in nutrient concentration between streams or due to geographical distribution patterns since the two streams were located in different regions.

This higher microbial activity on oak leaves in the nutrient enriched than in the reference stream lead probably to an increase in quality of the substrate as previously observed for other litter types (GULIS & SUBERKROPP, 2003b). Not surprisingly, oak leaves incubated in

nutrient enriched streams supported higher abundance and richness of invertebrates than leaves in reference streams (ROBINSON & GESSNER, 2000; NIYOGI *et al*., 2003; PASCOAL *et al*., 2003). The difference between stream types was higher for the poor quality oak leaves than for alder leaves that already have high nutrient content.

The present results show that decomposition and related parameters could be sensitive to organic pollution. The ratio of nutrient enriched/reference streams decomposition rate generally pointed out nutrient enriched streams as impacted, as predicted by GESSNER & CHAUVET (2002), being, however, more consistent when oak leaves in coarse mesh bags were used. This was also the assessment measure proposed by GESSNER & CHAUVET (2002) that best discriminated between enriched and reference streams. Both absolute decomposition rates of alder leaves and ratio of coarse/fine mesh bags *k* value were not helpful in assessing ecological stream integrity as they classified reference stream as impacted or, in the case of decomposition rates of alder leaves in fine mesh bags, enriched streams as not impacted. It seems therefore that absolute *k* values can be very variable in terms of dependence to general environmental factors.

If decomposition is to be used as a functional parameter, how comparable are the results with measurements of structural parameters of the benthic macroinvertebrate community? Only the biotic index IBMWP and 14 metrics (out of 45; related to Plecoptera and Oligochaeta) applied to the benthic macroinvertebrate community were useful discriminating between reference and nutrient enriched streams (paired t test). The order Plecoptera includes taxa (e.g., Leuctidae, Nemouridae) that besides being shredders are considered to be sensitive to organic pollution, as opposite to Oligochaeta (ALBA-TERCEDOR, 1996; TACHET *et al.*, 2000). Other studies have applied metrics to assess ecological quality of streams with contrasting water chemistry and found them highly variable with sampling date (discharge) which confounds its discriminatory ability (MORAIS *et al*., 2004, but see PINTO *et al*., 2004). The biotic index IBMWP was significantly higher in reference than in enriched streams, however, classified all stream as not polluted (class I, from I–V). It is plausible that climate and geographic constrictions dictating community composition and diversity may affect the output of biotic indices, with high productive, species rich zones having naturally higher IBMWP scores than less diverse areas. If this is the case, biotic indices seem less robust than functional approaches to assess environmental health.

Discriminating metrics were more correlated with SRP than with NO_3-N concentration in water, which points to the possibility that phosphorus might be more important than

nitrogen in its stimulation effect not only of decomposers but also of invertebrates. When streams were ordinated (CA) based on their benthic macroinvertebrate communities, reference and nutrient enriched streams were discriminated and appeared distributed along axis 1 which was significantly correlated with SRP concentration in water. This was mainly due to the exclusive presence of *Amphinemura* sp. and Perlidae stoneflies and Scirtidae coleopterans in reference streams and Caenidae and Ephemeridae mayflies in nutrient enriched streams. Also, Heptageniidae mayflies, stoneflies, Beraidae caddisflies, Hydraenidae coleopterans, Aeshidae dragonflies and Planariidae flatworms were more abundant in reference than in nutrient enriched streams while the opposite was true for Ephemerellidae mayflies, Sericostomatidae caddisflies, Elmidae coleopterans and Erpobdellidae leeches. This distribution of families through streams was not always consistent with their scores in the biotic index IBMWP, which, for instance, considers the families Ephemeridae and Sericostomatidae as very sensitive to organic pollution (score of 10; ALBA-TERCEDOR, 1996). The distribution of streams/reaches (multivariate analysis) along a pollution gradient was also found in other studies (PASCOAL *et al*., 2003; MORAIS *et al*., 2004; PINTO *et al*., 2004), reinforcing the sensitivity of the invertebrate community to nutrient enrichment of stream water.

In conclusion, decomposition rates were better discriminating nutrient enriched from reference streams than indices and metrics based on the benthic macroinvertebrate community, and only comparable with multivariate analysis of benthic community structure. Oak decomposition rates were more powerful than alder decomposition rates to discriminate between stream types. Leaf decomposition in coarse mesh bags was also more powerful to distinguish between enriched and reference streams than decomposition in fine mesh bags, probably because decomposition in coarse mesh bags reflects the response of the entire community to nutrient enrichment which is important since invertebrates (shredders in particular) are major intervenients in litter decomposition. Therefore, the sensitivity of decomposition rates, in particular those of oak in coarse mesh bags, to nutrient enrichment of stream water made them valuable in assessing stream ecological health as proposed by GESSNER & CHAUVET (2002).

However, for decomposition rates of leaf substrates incubated in mesh bags to be used as an assessment tool of environmental quality it is necessary to rule out physical fragmentation by current and suspended sediments as a cause for differences in mass loss between streams. This might be achieved by allocating mesh bags in reaches with similar physical characteristics. Also, care should be taken when using decomposition rates to assess

eutrophication since other disturbances to the aquatic system like modification of the riparian zone (reviewed by GRAÇA *et al*., 2002), acidification (DANGLES *et al*., 2004) and metal contamination (NIYOGI *et al*. 2001) can affect decomposition rates in an opposite way to nutrient enrichment and therefore mislead the results of the assessment.

Nevertheless, decomposition rates are a promising tool to assess environmental quality since this is an integrative measurement of the biotic and abiotic characteristics of the aquatic system (GESSNER & CHAUVET, 2002). In addition, it is a low cost and low time consuming methodology and requires low technical expertise in comparison with measures of structural parameters.

Although some parameters associated with fungi and invertebrates were also useful discriminating polluted from reference streams there is still a need for more studies since those parameters were measured only in one pair of streams. However, parameters associated with fungi and invertebrates would always be more expensive, time consuming and would require high technical and taxonomic expertise.

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CHAPTER III

WHOLE-STREAM NITRATE ADDITION AFFECTS LITTER DECOMPOSITION AND ASSOCIATED FUNGI BUT NOT INVERTEBRATES

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Photos:

Margaraça Stream, Margaraça Forest, Açor Mountain, Portugal

Nitrogen addition apparatus

Senescent air-dry alder (*Alnus glutinosa* (L.) GAERTNER) leaves

Senescent air-dry oak (*Quercus robur* (L.)) leaves

Balsa (*Ochroma pyramidale* (CAV. EX LAM.) URB.) veneers

WHOLE-STREAM NITRATE ADDITION AFFECTS LITTER DECOMPOSITION AND ASSOCIATED FUNGI BUT NOT INVERTEBRATES

ABSTRACT

The effect of whole-stream nitrate enrichment on decomposition of 3 substrates differing in nutrient quality (alder and oak leaves and balsa veneers) and associated fungi and invertebrates was assessed. During the 3-month nitrate enrichment of a headwater stream in central Portugal, litter was incubated in the reference site (mean 82 μg NO_3-N L^{-1}) and 4 enriched sites along the nitrate gradient (214–983 μg NO_3-N L^{-1}). A similar decomposition experiment was also carried out in the same sites at ambient nutrient conditions next year (33–104 μg NO_3-N L^{-1}). Decomposition rates and sporulation of aquatic hyphomycetes associated with litter were determined in both experiments, whereas N and P content of litter, associated fungal biomass and invertebrates were followed only during the nitrate addition experiment. Nitrate enrichment stimulated decomposition of oak leaves and balsa veneers, fungal biomass accrual on alder leaves and balsa veneers and sporulation of aquatic hyphomycetes on all substrates. Nitrogen concentration in stream water showed a strong asymptotic relationship (Michaelis-Menten-type saturation model) with temperature-adjusted decomposition rates and % initial litter mass converted into aquatic hyphomycete conidia for all substrates. Fungal communities did not differ significantly among sites but some species showed substrate preferences. Nevertheless, certain species were sensitive to nitrogen concentration in water by increasing or decreasing their sporulation rate accordingly. N and P content of litter and abundances or richness of litter-associated invertebrates were not affected by nitrate addition. It appears that microbial nitrogen demands can be met at relatively low levels of dissolved nitrate, suggesting that even minor increases in nitrogen in streams due to e.g. anthropogenic eutrophication may lead to significant shifts in microbial dynamics, plant litter decomposition and ecosystem functioning.

Key-words: aquatic hyphomycetes, biomass, leaves, nitrogen, sporulation, wood

INTRODUCTION

The primary source of energy and carbon in small woodland streams is allochthonous organic matter provided by trees from the riparian zone (FISHER & LIKENS, 1973; VANNOTE *et al*., 1980; WEBSTER & MEYER, 1997) mainly in the form of leaves and wood. Both microorganisms and shredding macroinvertebrates are important players in the decomposition of this organic matter and their relative importance varies significantly among streams (e.g. HIEBER & GESSNER, 2002 and references therein). Microbial decomposition in streams is primarily driven by aquatic fungi (KAUSHIK & HYNES, 1971; GESSNER & CHAUVET, 1994; BALDY *et al*., 1995) that incorporate leaf carbon into their mycelial biomass and conidia, mineralize it and also enhance leaf consumption by shredders through leaf litter conditioning (BÄRLOCHER & KENDRICK, 1981; SUBERKROPP, 1992; GRAÇA, 2001). Since fungi can obtain nutrients from both the substrate and surrounding water (SUBERKROPP, 1998), both litter quality (GESSNER & CHAUVET, 1994; ROYER & MINSHALL, 2001; DÍEZ *et al*., 2002; STELZER *et al*., 2003) and nutrient concentrations in water (SUBERKROPP & CHAUVET, 1995; NIYOGY *et al*., 2003; PASCOAL *et al*., 2001, 2003) affect their activity and hence decomposition.

Nutrient enrichment generally stimulates decomposition of plant litter in streams and associated microorganisms (e.g. ELWOOD *et al.* 1981; GRATTAN & SUBERKROPP, 2001; GULIS & SUBERKROPP, 2003) and the stimulation effect is more pronounced for low quality (i.e. low nutrients and high lignin) substrates (STELZER *et al*., 2003; GULIS *et al*., 2004). However, in streams where the certain inorganic nutrient (i.e. N or P) is not limiting, further increases in its concentration in water may not enhance litter decomposition or activity of associated microorganisms (GRATTAN & SUBERKROPP, 2001; ROYER & MINSHALL, 2001). Furthermore, in case of organic pollution (e.g. sewage effluents), the oxygen concentration in water may drop significantly, which can lead to decreased activity, abundance and/or diversity of invertebrates and microbial decomposers, resulting in slower decomposition rates (PASCOAL *et al*., 2001).

The numbers, diversity and biomass of invertebrates colonizing leaves have been related to nutrient concentration in stream water (ROSEMOND *et al*., 2002; NIYOGI *et al*., 2003; PASCOAL *et al*., 2003). The higher abundances of invertebrates associated with submerged leaf litter were reported in fertilized stream-side channels in comparison to the control (PEARSON & CONNOLLY, 2000) and in fertilized *vs*. control bags that corresponded to accelerated litter mass loss (ROBINSON & GESSNER, 2000). This could be a result of an increased fungal

biomass associated with leaf litter (as reviewed by GRAÇA, 2001), but ROBINSON & GESSNER (2000) did not report such increases.

Field studies assessing the effect of nutrients on litter decomposition and associated parameters relied on comparisons of the reference unit(s) with (a) already existing high nutrient streams (SUBERKROPP & CHAUVET, 1995; DÍEZ *et al*., 2002; NIYOGY *et al*., 2003) or (b) experimentally enriched litter bags (ROBINSON & GESSNER, 2000; ROYER & MINSHALL, 2001), flow-through (GRATTAN & SUBERKROPP, 2001) or stream-side channels (PEARSON & CONNOLLY, 2000), whole streams (ELWOOD *et al*., 1981; NEWBOLD *et al.*, 1983; GULIS & SUBERKROPP, 2003; STELZER *et al*., 2003; GULIS *et al.*, 2004) or catchments (CHADWICK & HURYN, 2003). A few studies dealt with decomposition and associated parameters along a nutrient gradient, within several streams (SUBERKROPP & CHAUVET, 1995; ROSEMOND *et al*., 2002; NIYOGY *et al*., 2003) or within the same polluted river (PASCOAL *et al*., 2003). However, different streams or reaches several kilometers apart may vary not only in nutrient content but also in other physical, chemical or biological parameters.

In this study, it was attempted to avoid such confounding effects by creating an experimental nitrate gradient within a short reach in an N-limited, first order forested stream in central Portugal and sampling at several sites within this reach (1–12x ambient N concentration). We studied the effect of nitrate enrichment on decomposition of three substrate types differing in initial nutrient quality and on associated fungal and invertebrate parameters. Specifically, by creating a nitrate gradient it was attempted to address the quantitative aspects of the response of decomposition and associated parameters to enrichment, such as the shape of the response curve or the existence of threshold or saturation phenomena within the nitrate concentration range.

METHODS

Study site

The study stream was located in the Margaraça Forest (Açor Mountain, Central Portugal, 40º13' N; 7º56' W). The forest is a 50-ha protected area with NNW exposure, *ca*. 25º slope and altitude ranging from 600 to 850 m a.s.l. The area has schistous soils and is covered by old growth deciduous forest composed mainly by *Castanea sativa* MILLER, *Quercus robur* L., *Arbutus unedo* L. and *Ilex aquifolium* L. (PAIVA, 1981). The same trees dominated the riparian zone of the experimental first order stream providing a closed canopy.

The experimental stream section was *ca.* 270 m long, 0.5–2 m wide and had a maximum depth of about 10 cm. It is a softwater, circumneutral stream with relatively low dissolved inorganic nitrogen concentration but high soluble reactive phosphorus (SRP), which could be explained by underlying geology. Some physico-chemical parameters of the stream during the experiments are presented in **Table 1**. It was not found any obvious trends in nutrient concentrations along the 270 m experimental reach during the decomposition experiment under ambient conditions or just before nitrate addition started (see below). For more information about the stream see ABELHO & GRAÇA (1998).

Table 1. Physico-chemical characteristics of the study stream during decomposition experiments at ambient nutrient conditions and nitrate addition. Mean values with ranges in parentheses are given.

Parameter	Ambient nutrients experiment	Nitrate addition experiment
Discharge (L s^{-1})	0.9 (0.7–1.6)	1.9 (0.7–3.0)
Temperature (ºC)	7.9 (2.8–13.6)	9.6 (7.5–11.7)
Conductivity (µS cm^{-1})	60 (50–63)	62 (53–71)
pH	7.3 (7.0–7.7)	6.8
Alkalinity (mg $CaCO_3$ L^{-1})	–	31
SRP (µg L^{-1})	91 (43–216)	71 (57–86)
NH_4-N (µg L^{-1})	–	<10
NO_3-N (µg L^{-1})	65 (7–197)	82 (32–123)*

Nitrate gradient

Nitrate enrichment experiment was designed to (1) achieve the target NO_3-N concentration of 1000 µg L^{-1} in the most enriched site (i.e. *ca.* 12x the ambient concentration), (2) obtain a gradient of nitrogen concentration downstream from the addition point and (3) achieve the nitrate concentration just above the ambient in the most downstream site. Continuous enrichment of stream water (October 9, 2003–January 30, 2004) was achieved by dripping concentrated filtered $NaNO_3$ solution from a battery of five 5 L glass Mariotte bottles. Bottles were refilled weekly and the dripping rate was corrected according to the instantaneous discharge (see below). Complete mixing of the solution with stream water was ensured by water passing through a 4 m long pipe just downstream from the addition point.

Litter bags (see below) were incubated in 5 sites (each 3–5 m long): the reference site just upstream from the addition point (R) and 4 enriched sites downstream from the addition point, at 10 (N1), 100 (N2), 220 (N3) and 255 m (N4). A good contrast in nitrate concentrations among these relatively closely spaced stream sites was achieved (**Fig. 1**) because of presumably high biotic nitrate uptake and a dilution affect (discharge increased by *ca.* 40 % from R to N4). In autumn 2004, litter bags were incubated at the same sites but

nutrients were not added (**Fig. 1**) and this decomposition experiment is further referred to as "ambient nutrient condition" experiment.

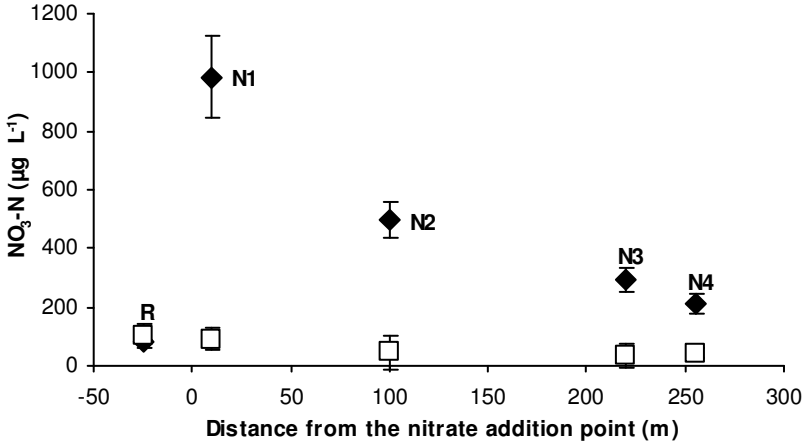

Figure 1. Nitrogen concentration (mean±1SD) in stream water during decomposition experiments at ambient nutrient concentrations (□, *n*=12) and during the nitrate addition (◆, *n*=9) in the reference (R) and enriched (N) sites.

Water parameters

Water samples for nutrient analyses were taken weekly during the decomposition experiments. Water was filtered in the field through glass fiber filters (Millipore APFF), transported to the laboratory on ice and frozen until analyzed. Additional unfiltered water samples were taken during the nitrate addition only and analyzed within 24 h for ammonium and alkalinity (APHA, 1995). Nitrate and ammonia were determined by ion chromatography (Dionex DX-120), SRP by the ascorbic acid method (APHA, 1995) and alkalinity by titration to an end pH of 4.5 (APHA, 1995).

Water temperature was continuously monitored at the reference site (both years) and additionally at N4 site during the nitrate addition experiment with Optic StowAway temperature probes (Onset Computer Corp.) that recorded temperature every 30 min. Stream water conductivity (WTW LF 330) and pH (Jenway 3310.) were measured weekly. Stream discharge was determined weekly (to adjust the nitrate addition rate; see above) by short term (10–15 min.) conservative tracer (250 g NaCl L^{-1}) release, using the following formula: D = ($[Cl^-]$*release rate)/((Condf – Condi)*(0.32 – 0.15)), where D is the discharge in L s^{-1}, $[Cl^-]$ is the Cl^- concentration in mg L^{-1} (=151709), release rate is the release rate of NaCl solution in L s^{-1}, Condf is the conductivity after Cl^- concentration reached a plateau in stream water in µS cm^{-1}, Condi is the background conductivity in µS cm^{-1}, 0.32 is the slope in the conductivity-

chloride concentration regression equation and -0.15 is the intercept in the same equation (MULHOLAND *et al*., 1994; WEBSTER & EHRMAN, 1996).

Litter bags and decomposition

Three substrate types were used in the decomposition experiments: leaves of alder (*Alnus glutinosa* (L.) GAERTNER) and oak (*Quercus robur* L.) in batches of 3.0–3.5 g per bag and balsa veneers (*Ochroma pyramidale* (CAV. EX LAM.) URB.), 2 veneers 10 x 7 x 0.1 cm (1.8–4.6 g) per bag. Leaves were collected, from the same stand of trees in October–November 2003, just after abscission, air-dried and stored until needed. Balsa veneers were purchased from a local supplier.

Substrates were deployed in the stream in coarse mesh bags (10 x 15 cm, 10 mm mesh size) on November 5, 2003 (nitrate addition experiment) and on October 17, 2004 (ambient nutrient condition experiment). Twenty bags of each substrate type were affixed to the streambed with nails at each experimental site. Four to six extra bags of each substrate type were taken to the stream and brought back to the laboratory the same day to determine initial ash free dry mass (AFDM, see below) taking into account losses due to handling. Alder was sampled weekly over 5 weeks, oak and balsa at 2–3 weeks interval over 12–15 weeks. At each sampling date, 4 replicate bags per substrate were retrieved from each site, placed in individual zip-lock bags and transported to the laboratory on ice for processing (within 24 h after collection). Each sample was gently rinsed with distilled water on top of a 0.5 mm mesh sieve to remove sediments and invertebrates (see below) and 2 sets of 5 disks each (12 mm diameter) were punched out with a cork borer for fungal biomass and sporulation rates determination (see below). The remaining material was oven dried at 105 ºC for 24–48 h, weighed, ashed at 550 ºC for 4–6 h and reweighed to determine AFDM remaining.

Nitrogen and phosphorus in litter

On day 0 (alder, oak and balsa), 25 (alder), 55 (oak) and 86 (balsa) of the nitrate addition experiment, a subsample of leaf material or veneer was taken for nitrogen and phosphorus analyses. Nitrogen was extracted from 1.0–2.5 mg ground oven-dry material (105 ºC, 24–48 h) by acid digestion (96 % H_2SO_4 plus $CuSO_4$) on a hot plate (reduction to ammonia). The extract was neutralized, diluted, filtered and the absorbance was read at 630 nm after incubation with sodium nitroprussid and sodium hypochlorite reagents for 1 h (FLINDT & LILLEBO, 2005). Phosphorus was extracted from 4–5 mg of combusted leaf or wood material (550 ºC, 4–6 h) by acid digestion (37 % HCl) on a hot plate. The extract was

diluted, filtered and phosphorus was determined by the ascorbic acid method (FLINDT & LILLEBO, 2005). Results were expressed as % N and % P of the remaining litter AFDM.

Fungal biomass

Sets of 5 leaf or veneer disks collected on day 0, 18 (alder), 40 (oak) and 47 (balsa) of the nitrate addition experiment were used to estimate fungal biomass associated with decomposing litter from ergosterol concentration. Samples were collected during periods of supposed peak fungal biomass according to previous experience. The disks were stored in 10 mL of KOH in methanol solution (8 g L^{-1}) at –18 ºC until extraction. Lipid extraction and saponification was carried out at 80 ºC for 30 min., with stirring. The extract was then purified by solid phase extraction (Waters Sep-Pak Vac RC tC_{18} cartridges) (GESSNER & SCHMITT 1996; GESSNER, 2005). Ergosterol was quantified by high performance liquid chromatography (HPLC) by measuring absorbance at 282 nm. The HPLC system (Dionex DX-120) was equipped with the Brownlee reverse phase C_{18} column (Applied Biosystems) maintained at 33 ºC. The mobile phase was 100 % methanol and the flow rate was set to 1.5 mL min^{-1}. Ergosterol was converted into fungal biomass using a conversion factor of 5.5 µg ergosterol mg^{-1} fungal dry mass (GESSNER & CHAUVET, 1993). Results were expressed as mg fungal biomass g^{-1} AFDM.

Aquatic hyphomycete sporulation

A set of 5 leaf or veneer disks was placed in 100 mL Erlenmeyer flasks containing 25 mL of filtered (Millipore APFF) water from the corresponding stream site. Incubations were carried out on an orbital shaker (100 rpm) for 48 h at 15 ºC to induce sporulation by aquatic hyphomycetes. The conidia suspensions were decanted in 50 mL centrifuge tubes, flasks rinsed twice, and conidia fixed with 2 mL of 37 % formalin to be counted and identified later. The corresponding disks were saved and their AFDM was determined as described above. When preparing slides for conidia identification, 100 µL of 0.5 % Triton X-100 solution were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot of the suspension was filtered (Millipore SMWP, 5 µm pore size). Filters were stained with cotton blue in lactic acid (0.05 %) and spores were identified and counted with a compound microscope at 200x. Sporulation rates were expressed as number of conidia released mg^{-1} AFDM day^{-1}.

Macroinvertebrates

Litter bag invertebrates were sampled only during the nutrient addition experiment. After rinsing each litter sample, invertebrates retained on sieve (0.5 mm mesh) were collected and stored in 70 % ethanol. Identification was done to genus or species level when possible, except for Oligochaeta and some Diptera (family and subfamily or tribe, respectively), and for Hydracarina and Ostracoda (presence). The invertebrates were classified into two categories: shredders and non-shredders (TACHET *et al*., 2002).

DATA ANALYSIS

Decomposition rates, *k*, were calculated by linear regression of ln transformed data (negative exponential model $M_t = M_o \cdot e^{-kt}$, where M_o is the initial mass, M_t is the remaining mass at time t, and *k* is the decomposition rate). To account for rather small, but potentially important, differences in temperature between years, decomposition rates in degree days were also calculated by replacing time (t) by the sum of mean daily temperatures accumulated by the sampling day. Decomposition rates for each substrate type were compared by ANCOVA with site as categorical variable and time as continuous variable, followed by Tukey's test (ZAR, 1999). Relationships between nitrogen concentrations in water and decomposition rates were assessed by Michaelis-Menten-type saturation model (nonlinear curve fitting) and linear regression. Nitrogen and phosphorus content of plant litter and associated fungal biomass were compared among sites by 1-way ANOVA followed by Tukey's test.

Sporulation rates of aquatic hyphomycetes and invertebrate and shredder abundances were compared among sites by 2-way ANOVA with stream site and time as categorical variables, followed by Tukey's test. The mass of conidia produced at each sampling date was estimated by multiplying sporulation rate of each species by specific mass of single conidium that were either published (CHAUVET & SUBERKROPP, 1998) or calculated from published biovolume data (BÄRLOCHER & SCHWEIZER, 1983) and summing up. Cumulative conidial production by the end of decomposition experiments (in mg of conidial AFDM per mg of initial litter) was calculated by summing up values of daily production at each sampling date and linearly approximated values for each day between sampling dates. Conidial production data at each sampling date were resampled with replacement (1000 times) to calculate and compare the percentage of initial litter mass converted into conidia at each stream reach and estimate 95 % confidence limits. The relationship between the nitrogen concentration in water and the percentage of litter converted into conidia was assessed by Michaelis-Menten-type

saturation model and linear regression. Multidimensional scaling (MDS) based on Bray-Curtis similarity matrix of mean abundances of aquatic hyphomycete conidia was performed for 75 samples (5 sites x 3 substrate types x 5 sampling dates) with PRIMER 6 software (CLARKE & GORLEY, 2001). Analysis of similarities (ANOSIM) was used to assess differences in aquatic hyphomycete communities among substrates and sites (PRIMER 6; CLARKE & GORLEY, 2001). Relationships between nitrogen concentrations in water and mean abundances of aquatic hyphomycetes (based on spore production from both experiments) were assessed by Spearman rank correlation. Statistical analyses were done with STATISTICA 6 unless otherwise indicated.

RESULTS

Decomposition

During the ambient nutrient conditions, decomposition rates were generally similar to (alder) or even faster (oak and balsa) in the reference site than at most other sites (ANCOVA; **Table 2**). The relatively fast decomposition at site N4 during that season was probably caused by excessive mechanical abrasion during heavy rains due to suspended sediments in runoff from the unpaved road situated just upstream from site N4. In contrast, during the nitrate addition experiment, decomposition rates of oak at sites N1 and N4 and balsa veneers at N2, N3 and N4 were significantly higher than those in the reference site (ANCOVA followed by Tukey's test, $p=0.003$ for oak and $p<0.001$ for balsa; **Table 2**). Decomposition rates for alder were similar among the experimental sites (ANCOVA, $p=0.121$). There was no significant interaction (ANCOVA, $p=0.310$) between the effects of substrate and nitrate (site) while both site and substrate (ANCOVA, $p<0.001$) had significant effects on decomposition rates during the nitrate addition.

Temperature corrected decomposition rates (degree $days^{-1}$) of alder were similar between years in all sites except N4 (see above). Decomposition was not significantly different in the reference site between years for oak and balsa, while it was significantly faster during nitrate addition at stream sites N1 for oak and N2 and N3 for balsa (ANCOVA, $p<0.001$), suggesting that the observed differences were the result of manipulation and not intrinsic differences among reaches.

Table 2. Decomposition rates *k* (d^{-1}, 95 % CL in parentheses) of alder and oak leaves and balsa veneers incubated in the reference (R) and nitrogen enriched (N) sites during decomposition experiments at ambient conditions and nitrate addition. Comparisons were made among sites for each substrate type and experiment separately (ANCOVA followed by Tukey's test, different letters indicate significant differences ($p<0.050$) among slopes).

	Stream site	Alder		Oak		Balsa	
Ambient conditions	R	0.0309 (0.0036)	a	0.0091 (0.0002)	b	0.0077 (0.0005)	c
	N1	0.0293 (0.0020)	a	0.0079 (0.0002)	ab	0.0072 (0.0007)	c
	N2	0.0178 (0.0010)	a	0.0059 (0.0001)	a	0.0045 (0.0004)	b
	N3	0.0298 (0.0027)	a	0.0070 (0.0004)	ab	0.0021 (0.0002)	a
	N4	0.0741 (0.0048)	b	0.0136 (0.0007)	c	0.0076 (0.0004)	c
Nitrate addition	R	0.0319 (0.0019)	a	0.0103 (0.0005)	a	0.0064 (0.0004)	a
	N1	0.0416 (0.0031)	a	0.0146 (0.0011)	c	0.0088 (0.0004)	a
	N2	0.0328 (0.0014)	a	0.0099 (0.0004)	a	0.0098 (0.0003)	b
	N3	0.0363 (0.0030)	a	0.0116 (0.0005)	ab	0.0095 (0.0004)	b
	N4	0.0410 (0.0028)	a	0.0139 (0.0014)	bc	0.0104 (0.0004)	b

When decomposition data (degree $days^{-1}$) from both years (except N4 data at ambient condition, see above) were considered, the relationship between nitrate concentration in water and decomposition rate of all substrates was best explained by Michaelis-Menten-type saturation model (**Fig. 2**). Linear regressions of $\log_{10}(NO_3\text{-N})$ *vs*. decomposition rates were also significant for all substrates (p=0.011, 0.009 and 0.019 for alder, oak and balsa, respectively).

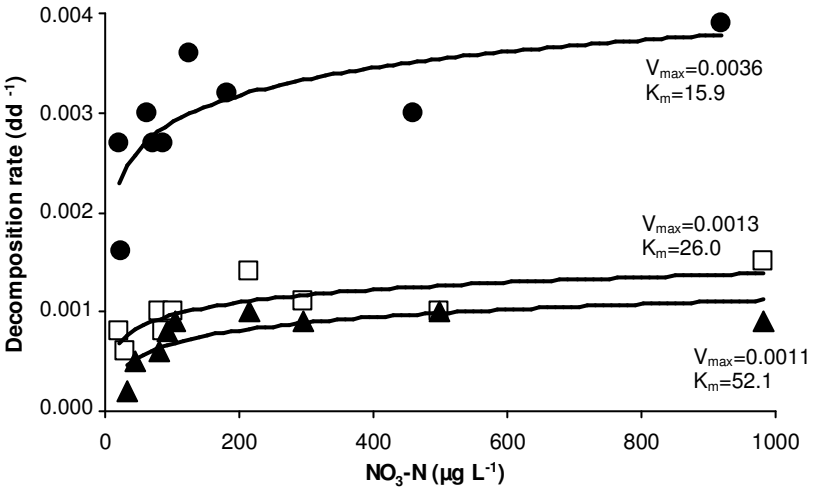

Figure 2. Relationship between the nitrate concentration in stream water and decomposition of alder (●) and oak (Í) leaves and balsa veneers (▲) during decomposition experiments at both ambient nutrient conditions and during nitrate addition. Data are fit into Michaelis-Menten-type model $V=(V_{max}\bullet[S])/(K_m+[S])$, where V_{max} is the maximum decomposition rate, K_m is the nitrate concentration at which half rate of decomposition is achieved, [S] is nitrate concentration. The model gave R^2 of 0.53, 0.58 and 0.78 for alder, oak and balsa, respectively, and $p<0.0001$ for all substrates.

Nitrogen and phosphorus in litter

Nitrogen content of alder leaves at day 25 (2.7–3.4 %), oak leaves at day 55 (1.5–2.1 %) and balsa veneers at day 86 (1.2–1.5 %) was generally higher than initial content (2.5, 1.0 and 0.3 %, respectively; **Fig. 3a**), suggesting microbial N immobilization at least for veneers. The highest nitrogen values associated with oak and balsa veneers were found in the site N2 while no differences among sites were detected for alder leaves.

Phosphorus content of alder leaves (0.11–0.14 %) and balsa veneers (0.13–0.16%) increased in comparison to the initial levels (0.09 and 0.01%, respectively), with a trend for higher values at the most N enriched sites (**Fig 3b**). Phosphorus content of oak leaves was similar across all sites (0.10–0.11 %) and did not increase in comparison to the initial level (0.11 %; ANOVA, $p>0.050$; **Fig. 3b**). No significant relationships were found between nitrogen or phosphorus content of all 3 substrates and nitrogen concentration in water during the nitrate addition experiment (linear regressions, $p>0.050$).

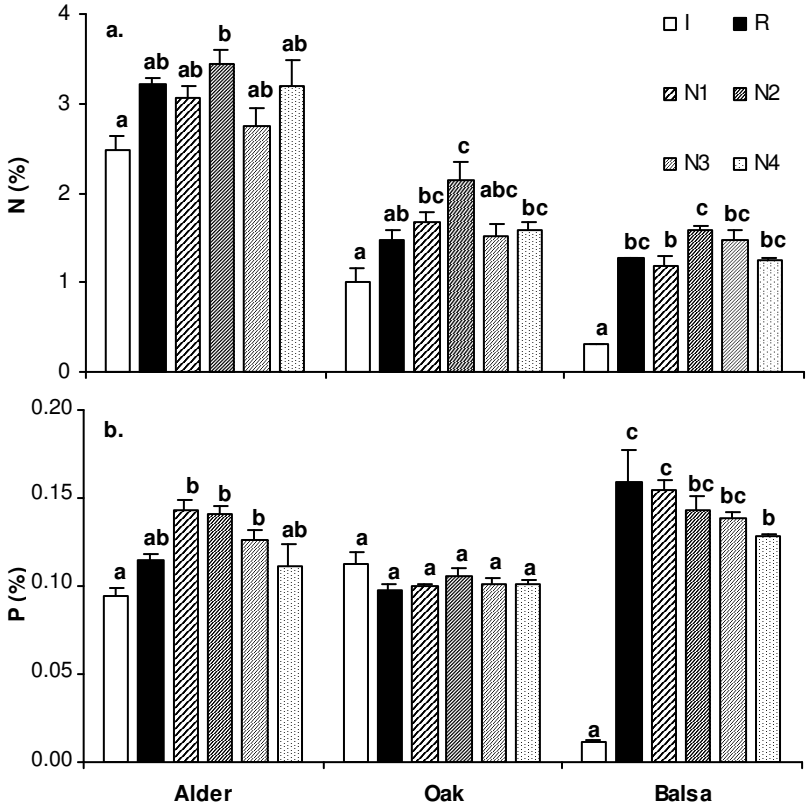

Figure 3. Nitrogen (**a**) and phosphorus (**b**) content (mean±1SE) of alder and oak leaves and balsa veneers at day 25, 55 and 86, respectively, in the reference (R) and nitrogen enriched (N) sites during nitrate addition experiment. Initial (I) N and P content of each substrate are also shown. Different letters indicate significant differences ($p<0.050$) among sites (1-way ANOVA followed by Tukey's test).

Fungal biomass

Fungal biomass associated with alder leaves at day 18 was significantly higher in the nitrate enriched sites (114–132 mg g^{-1} AFDM) than in the reference site (67 mg g^{-1} AFDM; ANOVA, $p<0.010$; **Fig. 4**), except for site N3 (91 mg g^{-1} AFDM; ANOVA, $p>0.050$). No significant difference among sites in fungal biomass associated with oak leaves at day 40 was found (117–134 mg g^{-1} AFDM; ANOVA, $p>0.050$). Fungal biomass associated with balsa veneers at day 47 was significantly higher at all nitrate enriched sites (136–177 mg g^{-1} AFDM) than in the reference site (69 mg g^{-1} AFDM; ANOVA, $p<0.001$). No significant relationships were found between fungal biomass associated with all 3 substrates and nitrogen concentration in water during the nitrate addition experiment (linear regressions, $p>0.050$).

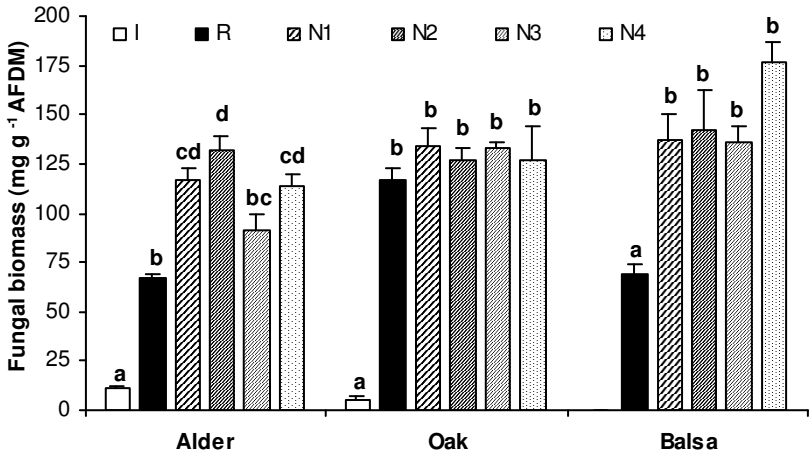

Figure 4. Fungal biomass (mean±1SE) associated with alder and oak leaves and balsa veneers at day 18, 40 and 47, respectively, in the reference (R) and nitrogen enriched (N) sites during nitrate addition experiment. Initial values for alder and oak are also provided. Different letters indicate significant differences ($p<0.050$) among sites (1-way ANOVA followed by Tukey's test).

Sporulation of aquatic hyphomycetes

Under ambient nutrient conditions sporulation rates of aquatic hyphomycetes were similar among all sites for oak (2-way ANOVA, $p=0.493$), but for alder and balsa, sites N2 and N3, respectively, differed from all other sites (2-way ANOVA followed by Tukey's test, $p<0.001$). During the nitrate enrichment, sporulation rates of aquatic hyphomycetes were higher in sites N1–N3 (alder), N1–N2 (oak) and all enriched sites (balsa) than in the reference site (2-way ANOVA, Tukey's test, $p<0.007$, **Fig. 5**). However, no differences in conidial production among the enriched sites (with the exception of N2 and N3 that had higher sporulation than N4 for alder leaves) were found.

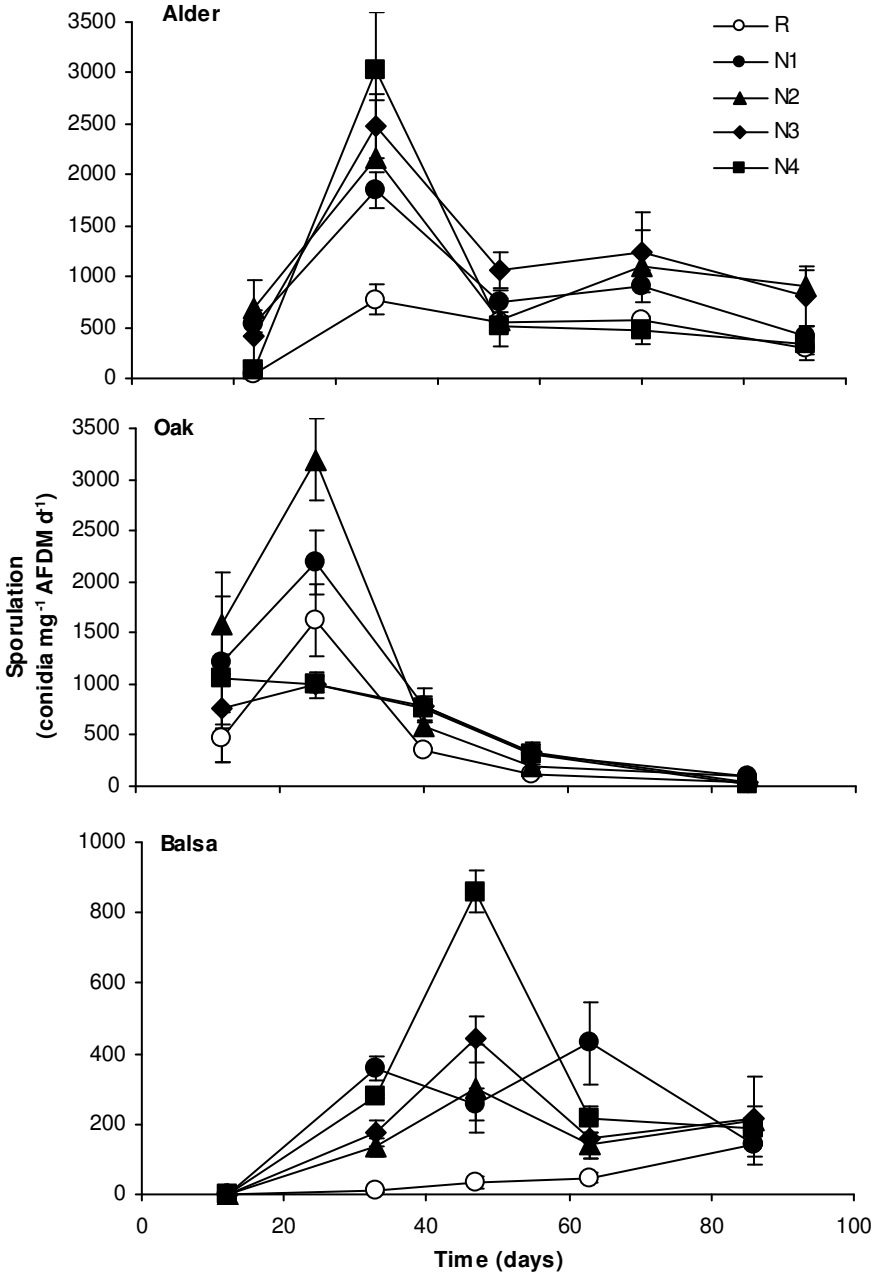

Figure 5. Sporulation rate of aquatic hyphomycetes (mean±1SE) associated with alder and oak leaves and balsa veneers in the reference (R) and nitrogen enriched (N) sites during nitrate enrichment experiment.

The percentage of initial alder and oak leaf litter and balsa wood converted into conidia by the end of experiment was higher in the enriched sites in comparison to the reference site in all instances during the nitrate addition experiment (**Fig. 6**). The relationship between nitrate

concentration in water and percentage of initial litter AFDM converted into conidia was best described by Michaelis-Menten-type saturation model (**Fig. 7**). Linear regression of $\log_{10}(NO_3\text{-N})$ *vs.* % initial litter AFDM converted into conidia was significant for oak only (p=0.069, 0.0003 and 0.146 for alder, oak and balsa, respectively).

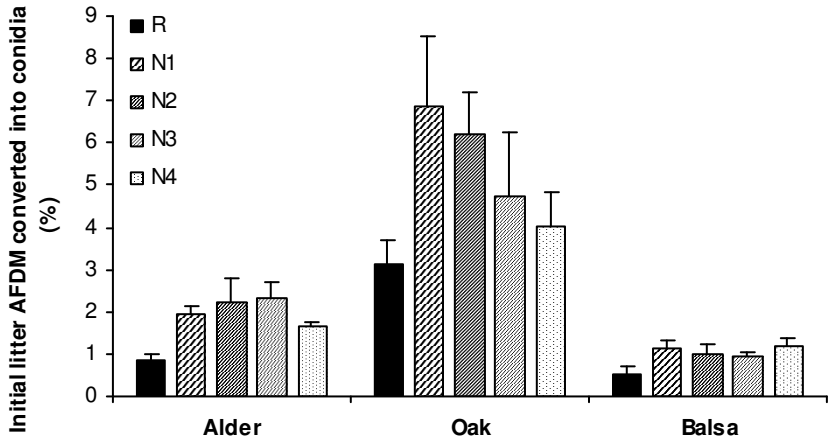

Figure 6. Percentage of initial AFDM (mean±95%CL) of alder and oak leaves and balsa veneers converted into aquatic hyphomycete conidia by day 33, 85 and 86, respectively, in the reference (R) and nitrogen enriched (N) sites during the nitrate addition experiment.

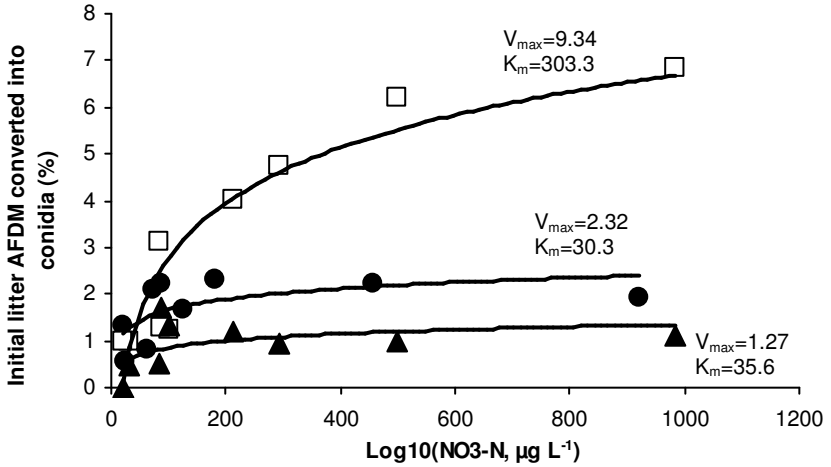

Figure 7. Relationship between the nitrate concentration in stream water and percentage of alder (●) and oak (□) initial leaf mass and balsa initial veneer mass (▲) converted into conidia by day 33, 85 and 86, respectively, of both ambient nutrient and nitrate addition experiments. Data are fit into Michaelis-Menten-type model (R^2=0.51, 0.92 and 0.39 for alder, oak and balsa, respectively, and p<0.001 for all substrates). Note that alder should not be directly compared with oak and balsa because of different exposure times, even though all substrates lost on average about 50–75 % of initial mass by the end of experiments.

A total of 23 species of aquatic hyphomycetes were identified in this study (**Table 3**). No apparent differences in total species richness were found among experiments or sites. *Tetrachaetum elegans* (43–83 % mean relative abundance) dominated fungal communities associated with alder leaves in all sites during the ambient condition experiment. *Lemonniera aquatica* (30 %) and *L. terrestris* (29 %) assumed the dominance in the reference site, while *Tetrachaetum elegans* (31–60 %) and *Articulospora tetracladia* (18–29 %) dominated on alder in the enriched sites during nitrate addition experiment. On oak leaves, *Tetrachaetum elegans* dominated in all sites during both experiments (35–79 %). Fungal community associated with balsa veneers was quite different from those of leaf litter. *Anguillospora crassa* (27–64 %) followed by *Clavariopsis aquatica* (17–50 %) dominated on wood in all sites during the ambient condition experiment and in the reference site during the nitrate addition. Their ranks switched (45–62 % for *C. aquatica* and 17–34 % for *A. crassa*) in the enriched sites during the nitrate addition experiment.

MDS ordination (**Fig. 8**) grouped samples by substrate type and not by stream site for both experiments (ANOSIM, p=0.001 and R=0.72 for substrate type and p=0.030 and R=0.07 for sites during ambient nutrient condition; p=0.001 and R=0.63 for substrate type and p=0.110 and R=0.05 for sites during the nitrate addition). Discrimination among samples by substrate type was mainly due to presence or absence of some species on a certain substrate (**Table 3**). Overall, the fungal community data was not useful in discriminating stream sites even when each substrate was considered separately. However, a few species were sensitive to nitrogen concentration in water and changed their sporulation rates accordingly (**Table 3**).

Macroinvertebrates

Abundance of invertebrates colonizing litter bags reached peaks of 28, 128 and 21 individuals g^{-1} AFDM for alder, oak and balsa, respectively (**Fig. 9**). Shredders represented 4–96 % of the total invertebrates. Overall, shredder abundance was low, typically less than 5 individuals g^{-1} AFDM. No significant differences were found among sites for alder leaves and balsa veneers for either total invertebrate or shredder abundances (2-way ANOVA, $p>0.339$). Oak leaves incubated at N3 site had lower abundance of invertebrates than leaves at R and N1 (2-way ANOVA followed by Tukey's test, $p<0.029$) while shredder abundance was higher at N1 sites than at R, N2 and N3 (2-way ANOVA followed by Tukey's test, $p<0.046$).

Table 3. Mean relative abundances (%, over all sampling dates) of aquatic hyphomycete conidia from alder and oak leaves and balsa veneers incubated in the reference (R) and nitrogen enriched (N) sites during decomposition experiments at ambient conditions (first line) and nitrate addition (second line). +, mean relative abundance <0.1 %

	Alder					Oak					Balsa				
Aquatic hyphomycete species	**R**	**N1**	**N2**	**N3**	**N4**	**R**	**N1**	**N2**	**N3**	**N4**	**R**	**N1**	**N2**	**N3**	**N4**
Alatospora acuminata, O**B**	2.3	3.9	7.2	5.0	2.3	4.0	2.4	7.7	3.1	6.1		0.1			
	2.3	4.6	4.5	5.4	4.7	2.8	1.4	1.7	1.3	1.7	+	0.7	0.3	0.3	0.1
Alatospora pulchella	0.6	0.9	2.0	0.1	0.1	1.7	0.3	1.5	0.2	1.6	+				
						0.5	0.2	0.2	0.3	0.1		0.1			
Anguillospora crassa, O**		0.1	0.1	0.4	0.2	0.7	0.8	0.4	0.9	0.3	61.3	56.8	63.5	26.8	53.8
	0.3		0.1	0.1	0.3	0.1				0.1	54.9	17.3	33.5	22.7	18.2
Anguillospora filiformis						0.1	0.1			0.4	2.0				
						0.1	+	0.1	+	0.1					
Articulospora tetracladia, A*	3.8	4.5	11.3	3.8	2.4	3.1	3.9	5.1	2.9	2.8					
	15.4	28.7	18.0	21.9	24.0	6.1	4.1	3.6	3.7	2.4		0.2			
Clavariopsis aquatica	0.1	0.1		0.1	0.4	5.5	2.3	2.2	3.8	9.0	17.2	22.9	36.0	50.4	25.5
	+	0.3	0.1	0.5	0.7	0.1	0.3	0.5	0.5	2.4	19.8	62.1	45.1	56.3	57.7
Culicidospora aquatica									+						
			0.1	0.1	0.3				0.1	0.1					
Dendrospora erecta									+						
Dendrospora cf. *fastuosa*								+							
Flagellospora curvula	7.9	5.9	21.2	2.6	3.7	0.8	0.5	7.1	0.4	0.1					
	+	0.8	1.8	9.1	20.6	+	+	0.3	0.3	1.2					
Heliscus lugdunensis, O*	0.2	0.1	1.4	+	+	+		+						1.4	
		0.5	0.2	0.1	0.4		0.3	0.1	0.1					+	
Lemonniera cf. *alabamensis*	6.5	2.5	1.5	0.2		2.5	0.4	0.3	0.1		2.7				
						7.7	1.4	0.3	0.3						
Lemonniera aquatica	29.9	13.0	3.1	2.5	0.5						1.4	1.6		0.9	0.3
Lemonniera terrestris, A*	5.7	5.9	4.9	0.9	1.5	2.7	1.5	1.6	1.1	0.2	15.4		0.1		
	29.3	13.5	6.3	16.1	6.4	7.1	2.3	1.7	2.5	1.2	18.8	16.4	20.0	17.6	19.2

Table 3. Continued

	Alder					Oak					Balsa				
Aquatic hyphomycete species	**R**	**N1**	**N2**	**N3**	**N4**	**R**	**N1**	**N2**	**N3**	**N4**	**R**	**N1**	**N2**	**N3**	**N4**
Lunulospora curvula	+	0.2	0.2	2.3	4.9				0.8	6.4					
				0.8	1.3			0.7	3.2	5.3					
Margaritispora aquatica, A*B*	1.0	0.7	3.1	0.9	0.7	3.2	4.0	3.1	3.5	4.3	0.1				0.1
	1.1	5.5	3.9	1.9	3.6	2.5	4.6	4.2	2.5	3.9	0.1	0.4	0.2	0.8	2.6
Stenocladiella neglecta	0.6	+	+				0.1	1.5		0.1					20.0
							+								
Tetrachaetum elegans, O*	68.3	70.3	43.2	82.6	78.3	52.6	67.7	49.0	57.1	35.1			+		
	19.8	31.1	60.0	39.8	35.8	68.1	78.9	78.6	76.3	66.4					
Tetracladium breve	+														
Tricladium chaetocladium	+	+	0.1		0.1	1.9	1.1	1.1	0.9	4.8					
			0.2	0.3	0.4	1.2	2.0	2.1	5.4	10.3			0.1		
Tricladium splendens	+	+		0.1	0.4	0.4	0.3	0.2	+	0.5	1.3	0.2	0.4	1.4	0.6
	+	0.2	+	0.1		0.2	0.1	0.1	0.1	0.1	4.7	0.5	0.9	1.1	1.8
Triscelophorus acuminatus	0.3	0.1	0.1	+	0.1		0.1		0.1						
	0.1				+	0.1	+	+	+						
Triscelophorus monosporus	0.4	0.9	0.2	+	0.2	10.2	4.0	1.4	1.3	11.2					
	+	0.2	0.2	0.1	0.1	0.5	0.5	1.4	0.3	0.2					
Unidentified (small irregularly branched)						1.1	1.5	6.5	9.1	11.7					
									+						
Unidentified tetraradiate	2.0	3.7	3.4	0.9	4.7	9.3	8.9	11.3	14.7	5.3					
	0.6		+		+	2.7	3.7	4.4	2.9	4.5	0.3	0.7		0.3	
Small sigmoid (<60 µm)															
							+								
Intermediate sigmoid (60–120 µm)															
	0.1					0.1									
No. species	17	17	15	15	15	15	16	16	17	15	8	4	5	4	5
	12	11	14	15	15	15	16	16	17	15	7	9	7	8	7

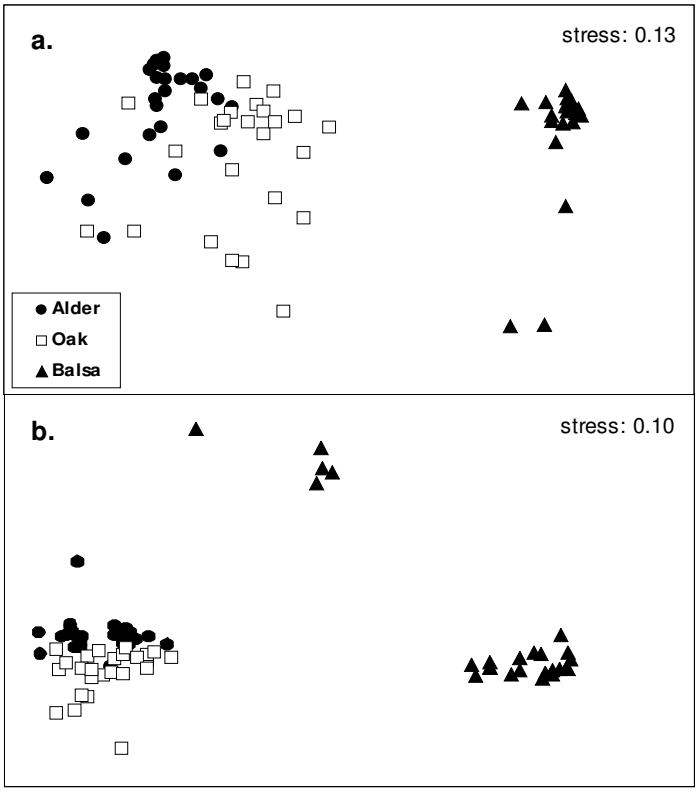

Figure 8. Ordination (MDS) of all samples (5 sites x 3 substrate types x 5 sampling dates) based on aquatic hyphomycete conidia abundances during decomposition experiments at both ambient nutrient concentrations (**a**) and nitrate addition (**b**). The stress was <0.20, the 2D ordination plot therefore faithfully represented the multi-dimensional relationships among the samples.

DISCUSSION

Decomposition of submerged plant litter and associated microbial activity and invertebrate colonization depend on both substrate qualities and a suite of stream characteristics. The design of this study favorably compares with many correlative studies where the effects in question can be confounded by uncontrolled variability of other parameters.

During the nitrate enrichment, decomposition rates of oak (but not alder) leaves and balsa veneers were generally higher than those under ambient nutrient conditions and the slowest decomposition was found in the reference site, suggesting that the observed differences were caused by the nitrate addition and not intrinsic differences among sites. Such stimulation of litter decomposition by increased nitrogen concentration in water has been shown by ELWOOD *et al.* (1981) for ammonium in an enrichment experiment and by

NIKOLCHEVA & BÄRLOCHER (2005) for nitrate in correlative study but was not found by GRATTAN & SUBERKROPP (2001) in a nitrate enrichment study. Overall, decomposition rates of alder leaves in this study were comparable to those reported in previous studies for the Iberian Peninsula (POZO *et al.* 1998; GRAÇA *et al*., 2001; PASCOAL *et al*., 2001, 2003, 2005; GONÇALVES *et al*., 2006), while oak showed faster decomposition than previously reported (MOLINERO *et al*., 1996). Decomposition of balsa veneers has not been previously studied in aquatic systems and it had relatively high decomposition rates in these experiments probably due, in part, to high surface to volume ratio and low density (SPÄNHOFF & MEYER, 2004). However, decomposition rates were lower than those reported by GULIS *et al.* (2004) for oak veneers in a nutrient enriched stream.

No obvious trends were found in decomposition rates between sites N1–N4 during the nitrate addition, suggesting that factors other than nitrate (e.g. mechanical fragmentation, invertebrate feeding, etc.) considerably affected decomposition rates at each particular site. Nevertheless, when *k* values from both experiments were pooled, significant relationships with nitrogen concentrations in water were found for all substrates. Michaelis-Menten-type model better explained our experimental data than linear regression of log transformed nitrate concentration *vs*. decomposition, even though both models were significant. Apparent nutrient saturation occurred at relatively low nitrate concentrations (half saturation constant K_m was less than 52 µg NO_3-N L^{-1} for all substrates), suggesting that even slight increases in nitrate concentration in water may lead to significant changes in decomposition rates. It is not surprising that decomposition of balsa veneers (low quality, low nutrient substrate) was the most nitrate limited (highest K_m), while decomposition of alder (high N substrate) was the least nitrate limited (lowest K_m). The magnitude of the response to nutrient enrichment has previously been shown to be driven in part by substrate C:N ratio (STELZER *et al*., 2003; GULIS *et al.*, 2004).

The absence of consistent differences in nitrogen and phosphorus content of all substrates among sites suggests that nutrient immobilization did not increase with higher nitrate concentration in water. The opposite was observed by GULIS & SUBERKROPP (2003) and GULIS *et al*. (2004) in a stream enriched by both N and P.

Fungal biomass associated with alder leaves and balsa veneers during the nitrate addition was significantly higher in the enriched sites than in the reference site. It is surprising that no similar response was observed for oak litter, especially in view of clearcut sporulation results (see below). Since fungal biomass often increases to a maximum and then declines, we

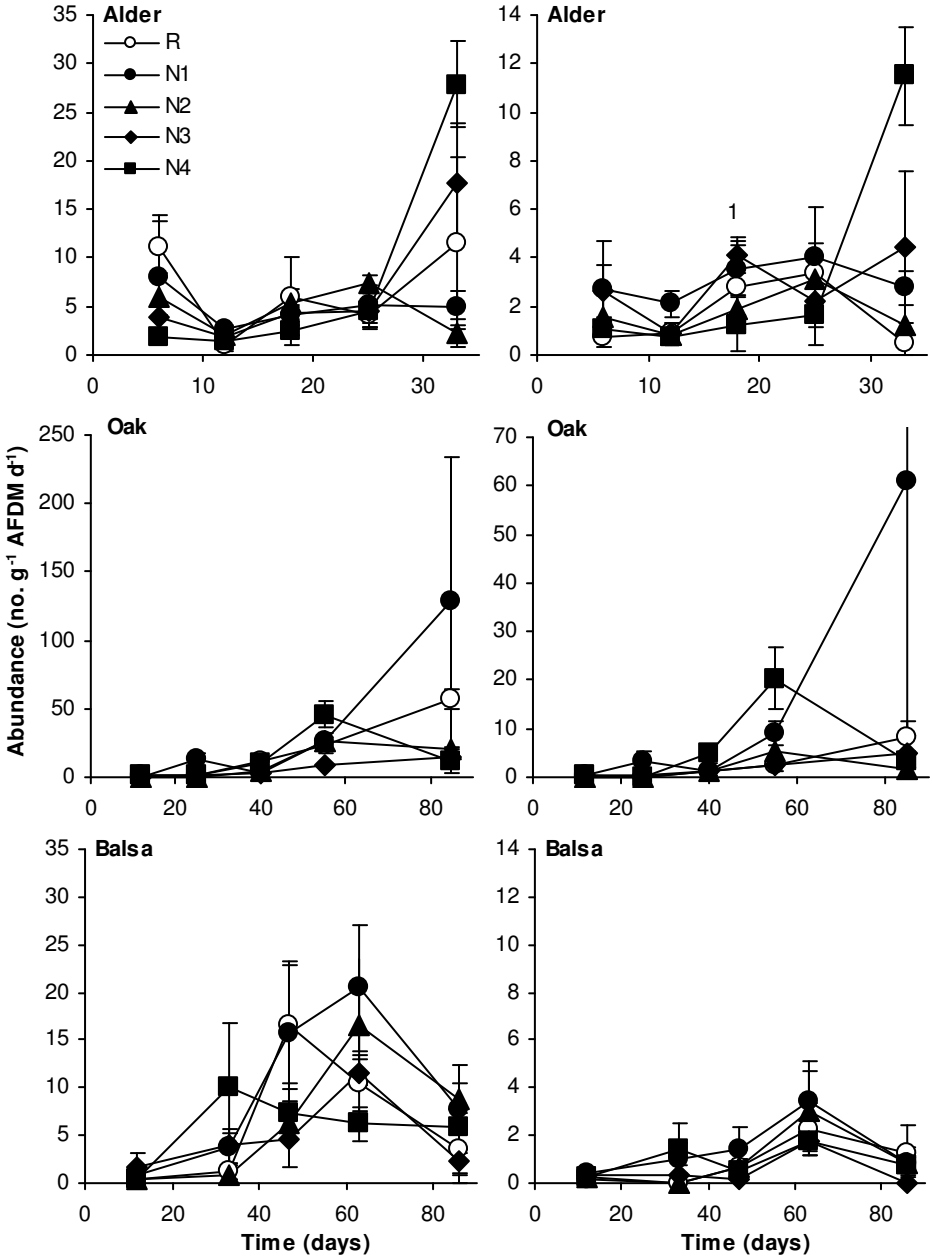

Figure 9. Abundance of total invertebrates and shredders (mean±1SE) associated with alder and oak leaves and balsa veneers in the reference (R) and nitrogen enriched (N) sites during nitrate addition experiment.

may have missed the ergosterol peak on oak litter because leaves from only one sampling date were analyzed. Experimental enrichment of stream water with nitrate has been reported to increase fungal biomass associated with yellow poplar litter (GRATTAN & SUBERKROPP, 2001)

but no effect has been found for 2 species of leaves and 5 types of wood in another experiment (STELZER *et al.*, 2003). In the latter case, fungi were probably P limited due to extremely low ambient SRP of stream water and, hence, N addition alone did not stimulate fungi; the stream used here, in contrast, had high natural P availability.

During the nitrate enrichment experiment, sporulation rates of aquatic hyphomycetes were stimulated at the most enriched sites, which is in agreement with previous correlative studies (SUBERKROPP & CHAUVET, 1995; NIKOLCHEVA & BÄRLOCHER, 2005), enrichment experiment (GRATTAN & SUBERKROPP, 2001) and laboratory studies (e.g. SUBERKROPP, 1998; SRIDHAR & BÄRLOCHER, 2000). In this study, the percentage of initial litter AFDM converted into conidia was also higher in the enriched sites than in the reference site for all substrates. Surprisingly, the extent to which cumulative conidial output was stimulated was similar among substrates. The ratio of the percentage of initial litter AFDM converted into conidia by the time of 60–70 % mass loss between the reference and the most enriched site (N1) for all substrates ranged between 2.2 and 2.3. This disagrees with the previous report of stronger stimulation of fungal sporulation by nutrient enrichment on lower quality than on high quality substrates (GULIS & SUBERKROPP, 2003). We also found strong asymptotic relationship between the nitrate concentration in water and efficiency of converting organic substrate into conidia. HURYN *et al.* (2002) also applied Michaelis-Menten-type saturation model to describe the relationship between nitrate in stream water and rate of leaf litter softening (penetrometry), which is thought to reflect microbial activity (SUBERKROPP *et al.*, 1983). The K_m values from this study are about an order of magnitude higher than those reported by HURYN *et al.* (2002) though they are not directly comparable because of different measured parameters and much higher SRP concentration in our stream that probably led to higher biotic N demand. Nevertheless, our K_m estimates are still relatively low to suggest that sporulation of aquatic hyphomycetes is quite sensitive to nitrate concentration in water and even low levels of eutrophication may cause profound changes in fungal activity and hence plant litter decomposition. The similarity between this saturation-type relationship for conidia production and the relationship between the nitrate concentration in water and litter decomposition rates suggests that aquatic hyphomycetes are indeed major players in organic matter processing in streams.

Only 23 species of aquatic hyphomycetes were identified from decaying litter in this study while 42 species were recorded from water samples from the same stream by BÄRLOCHER & GRAÇA (2002; the stream is referred to as Margaraça 1 there). The difference

in sampling technique and the fact that samples were collected over 12-month period in their study *vs*. only 3 months in autumn in this study could explain the difference in species richness. Overall, the fungal community data were not useful in discriminating among sites even though some fungal species were sensitive to nitrate concentration in water and increased or decreased their contribution to the total spore production. Similar sensitivity of certain fungal species to inorganic nutrients in water was found by GULIS & SUBERKROPP (2003) and PASCOAL *et al*. (2005). In this study, fungal communities were discriminated rather by the substrate type since some species showed clear preference towards specific substrate. Among the dominance species (> 5 % of conidia in a given substrate in a given site) *Articulospora tetracladia*, *Flagellospora curvula*, *Tetrachaetum elegans* and *Tricladium chaetocladium* preferred leaves while *Anguillospora crassa* and *Clavariospis aquatica* preferred wood veneers. The fungal assemblages colonizing balsa veneers were different from those colonizing leaves as they had lower number of species that were present in a different proportion in comparison to what was observed on leaves that led to a good separation in MDS analysis. Surprisingly, even the two leaf species were separated to some degree based on their fungal assemblages. The differences between fungal communities colonizing leaves *vs*. wood were previously reported by GULIS (2001) based on conidia occurrences while NIKOLCHEVA & BÄRLOCHER (2005) did not find strong effect of the substrate (including wood) on fungal communities using both traditional and molecular techniques.

Our nitrate enrichment did not affect total invertebrate or shredder abundances associated with decomposing litter, except for oak at site N1. This could be explained by the relatively short duration of nitrate exposure (1–3 months depending on the substrate). Apparently, at the concentration used, nitrate did not directly affect invertebrates causing drift or appreciable migration to or from the impacted sites. CROSS *et al*. (2005) found that long-term N and P enrichment of a headwater stream resulted in increased invertebrate abundances and production that was probably mediated by stimulated microbial activity. This experiment was too short to pick such an increase. Nevertheless some studies (PEARSON & CONNOLLY 2000, ROBINSON & GESSNER 2000) reported increased invertebrate abundances after a few months of fertilization.

Overall, nitrate enrichment of stream water appeared to stimulate plant litter decomposition, biomass and sporulation rate of aquatic hyphomycetes, which is in line with observations from correlative studies by SUBERKROPP & CHAUVET (1995) and NIKOLCHEVA & BÄRLOCHER (2005). In this experiment, the response of fungi and consequently

decomposition rate to nitrate was best described by the Michaelis-Menten-type saturation model, suggesting that responses to nutrient enrichment depend on absolute level of nutrient availability in streams. It appears that microbial nitrogen demands can be met at relatively low levels of nitrate (1 or 2 orders of magnitude lower than can be encountered in polluted streams) that suggests that even minor increases in dissolved nitrogen in streams due to e.g. anthropogenic eutrophication may lead to significant shifts in microbial dynamics, plant litter decomposition and ecosystem functioning.

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CHAPTER IV

EUCALYPTUS PLANTATIONS AFFECT FUNGAL COMMUNITIES ASSOCIATED WITH LEAF LITTER DECOMPOSITION IN IBERIAN STREAMS

FERREIRA V., ELOSEGI A., GULIS V., POZO J. & GRAÇA M A.S. 2006. Eucalyptus plantations affect fungal communities associated with leaf litter decomposition in Iberian streams. *Arch Hydrobiol* 166: 467–790

Photos:

Caramulo Mountain, Portugal

Lázaro Stream, Lázaro, Caramulo Mountain, Portugal

EUCALYPTUS PLANTATIONS AFFECT FUNGAL COMMUNITIES ASSOCIATED WITH LEAF LITTER DECOMPOSITION IN IBERIAN STREAMS

ABSTRACT

The replacement of diverse deciduous forests by eucalyptus plantations changes the timing, quality and quantity of litter inputs to streams, which has the potential to affect the activity of decomposers and thus ecosystem functioning. This study compared (a) the decomposition rate of alder and oak leaves incubated in streams running through deciduous forests and eucalyptus plantations in Spain and Portugal, (b) the activity (fungal biomass and sporulation) and diversity (species richness and Pielou's evenness index) of the associated fungal communities and (c) changes in N and P content of leaves. Alder and oak leaves decomposed at similar rates in both stream types and countries, with the exception of oak leaves in the Spanish eucalyptus stream, which decomposed faster than in the corresponding deciduous stream or in the Portuguese eucalyptus stream. This difference was attributed to physical fragmentation due to flooding and not to forest cover. Higher nitrogen and phosphorus content and higher fungal biomass and sporulation were generally found on leaves from eucalyptus rather than from deciduous streams. The higher fungal activity in eucalyptus streams was attributed to higher water temperature and benthic organic matter storage. The Spanish eucalyptus stream had higher species richness of aquatic hyphomycetes than the deciduous one (27 *vs.* 20) while in Portugal the opposite was true (16 *vs.* 20). Fungal community evenness was significantly higher on alder leaves in deciduous than in eucalyptus streams in Portugal while the opposite was true in Spain. The community structure (MDS analysis) discriminated both stream types in Portugal much better than it did in Spain. At least for Portugal, differences between stream types could be explained by higher litter diversity in the deciduous than in the eucalyptus stream. In conclusion, stream fungal communities in Portugal were more affected by eucalyptus plantations than in Spain. In both countries, fungal diversity and activity were more affected by eucalyptus plantations than decomposition rates of submerged litter. It is suggested therefore that, to mitigate this effect of eucalyptus plantations, deciduous trees could be planted on the river banks or, preferably, riparian strips of native vegetation should be left unmodified.

Key-words: aquatic hyphomycetes, biomass, ecosystem functioning, richness, sporulation

INTRODUCTION

Coarse particulate organic matter (CPOM) provided by riparian trees is the primary source of energy and carbon for aquatic communities in small woodland streams (FISHER & LIKENS, 1973; VANNOTE *et al.*, 1980). Aquatic fungi and invertebrate shredders are the key players in decomposition of this organic matter and its incorporation into aquatic food webs (HIEBER & GESSNER, 2002). As these organisms can be sensitive to the amount (WALLACE *et al.*, 1999; LAITUNG *et al.*, 2002) and quality (CANHOTO & GRAÇA, 1995, 1999; BÄRLOCHER & GRAÇA, 2002) of organic matter entering the streams, changes in the composition of the riparian forest can potentially affect both community structure and function of aquatic ecosystems.

Vast areas of deciduous forests have been converted into *Eucalyptus globulus* LABILL. plantations in the Iberian Peninsula. This substitution is highly profitable since *Eucalyptus globulus* has fast growth rates allowing prompt reforestation of burned areas and short rotation while timber is used by pulp and paper industry (reviewed by CANHOTO *et al.*, 2004). Eucalyptus plantations occupy 21 % of the forested area in Portugal (DIRECÇÃO GERAL FLORESTAS, 2005); data from Spain are less reliable because the Third National Forest Inventory is still in progress. However, eucalyptus plantations are known to represent around 12 % of total forest cover in the Atlantic regions of northern Spain (MINISTERIO DE MEDIO AMBIENTE, 2003). The replacement of diverse deciduous forests by evergreen eucalyptus trees changes the timing, quality and quantity of litter input to streams. In eucalyptus plantations, litter input occurs throughout the year, with a peak in summer, litter is less diverse and total input may be smaller and of lower nutritional quality (lower N and P content) than in native deciduous forests (reviewed by GRAÇA *et al.*, 2002; see also ABELHO & GRAÇA, 1996; POZO *et al.*, 1997a, b; MOLINERO & POZO, 2003, 2004). As a result, the inputs of nitrogen and phosphorus are also lower in these streams (POZO *et al.*, 1997a, b; MOLINERO & POZO, 2003). Despite the lower litter input, which peaks in summer during a period of low discharge, eucalyptus streams tend to store larger amounts of CPOM than deciduous ones (POZO *et al.*, 1997b; CANHOTO & GRAÇA, 1998; MOLINERO & POZO, 2003, 2004).

Comparisons of microbial communities between deciduous and eucalyptus streams in Spain and Portugal yielded contrasting results. BÄRLOCHER & GRAÇA (2002) reported that streams running through eucalyptus plantations in Portugal had lower diversity of aquatic hyphomycetes than streams running through deciduous forests. This could be explained in part by the lower diversity of CPOM. Some species of aquatic hyphomycetes have substrate

preferences (GULIS, 2001), so more diverse resources may support a higher number of species. However, in Spain, similar number of species was found in both stream types (CHAUVET *et al.*, 1997). Also, no differences in conidia concentration in water (BÄRLOCHER & GRAÇA, 2002), fungal biomass and nitrogen and phosphorus content of decomposing substrates (MOLINERO *et al.*, 1996; POZO *et al.*, 1998; DÍEZ *et al.*, 2002) were found between streams running through eucalyptus plantations and deciduous forests. Differences in diversity of aquatic hyphomycete communities may translate into differences in richness and diversity of invertebrates (ABELHO & GRAÇA, 1996) since shredders can discriminate among leaf patches colonized by different fungal species (ARSUFFI & SUBERKROPP, 1985, 1986, 1989; reviewed by SUBERKROPP, 1992).

Changes in litter seasonality, quantity and diversity, and their effect on invertebrates and aquatic hyphomycetes can potentially affect litter decomposition. However, the available information on this subject is contradictory. ABELHO & GRAÇA (1996) reported lower decomposition rates of *Castanea sativa* MILL. leaves in eucalyptus than in deciduous streams while no differences in decomposition of native and exotic leaf species and wood between both stream types were found in other studies (MOLINERO *et al.*, 1996; POZO *et al.*, 1998; BÄRLOCHER & GRAÇA, 2002; DÍEZ et al, 2002). If species richness and diversity of decomposers decline in eucalyptus streams without corresponding decrease in decomposition rates, then decomposers would be characterized by functional redundancy (WALKER, 1992).

There may be some differences in the effect of eucalyptus plantations on aquatic communities in Portugal and Spain, potentially explained by differences in climate (reviewed by GRAÇA *et al.*, 2002): precipitation is highly seasonal in Central Portugal whereas in Northern Spain it is more evenly distributed along the year. In this study, we investigated the decomposition of leaves of two native species (alder, *Alnus glutinosa* (L.) GAERTNER, and oak, *Quercus robur* L.) and associated fungal communities in two streams running through eucalyptus plantations and two streams running through deciduous forests, one pair in Portugal and the other in Spain. Alder and oak were selected because (a) they are close to the extremes of the decomposition rates continuum and (b) are common in the riparian area of most deciduous streams in both countries.

METHODS

Study sites

One stream running through a deciduous forest (deciduous stream) and one stream running through a eucalyptus plantation (eucalyptus stream) were selected in Central Portugal (Lousã and Caramulo mountains, respectively) and in Northern Spain (Cantabria) (**Table 1**). Chestnut (*Castanea sativa* MILL.) and oak (*Quercus* spp.) trees shaded the stream running through the deciduous forest in Portugal while in the eucalyptus plantation *Eucalyptus globulus* was the dominant species along the stream banks. Oak (*Quercus robur*) and alder *(Alnus glutinosa*) dominated the riparian corridor of the stream running through the deciduous forest in Spain while *E. globulus* was dominant along the eucalyptus stream but accompanied by young deciduous trees (oak, alder, hazelnut *Corylus avellana* L., ash *Fraxinus excelsior* L.) in the understory. Except for riparian vegetation, pairs of streams in both countries were chosen to be as similar as possible with respect to morphology, geology, substrate and physico-chemical characteristics (**Table 1**).

Water parameters

During the study period and in all streams, water temperature was recorded every hour (Portugal) or two hours (Spain) with temperature probes (Onset Optic StowAway in Portugal and ACR SmartButton in Spain), and conductivity (WTW LF 330) and pH (Jenway 3310 in Portugal and Hanna HI9025 in Spain) were measured 4–9 times. From each stream, 1 L of stream water was collected (4–9 times) in acid washed glass bottles (Portugal) or polyethylene flasks (Spain). Water was filtered through glass fiber filters in the field (Portugal, Millipore APFF) or transported in ice chests to the laboratory and then filtered (Spain, Whatman GF/F). An unfiltered subsample was promptly analyzed for ammonia while filtered subsamples for nitrate and soluble reactive phosphorus (SRP) analyses were frozen. Nitrate and ammonia concentrations were determined by ion chromatography (Dionex DX-120 in Portugal) or colorimetry (Autoanalyzer TRAACS 800 in Spain). SRP was determined by the ascorbic acid method (APHA, 1995). Alkalinity was measured on 3–9 occasions by titration to an end pH of 4.5 (APHA, 1995).

Litter bags and decomposition

In each country, alder (*Alnus glutinosa*) and oak (*Quercus robur*) leaves were collected from the same stand just after abscission in autumn 2002. Leaves were air-dried and stored dry until needed. On November 26 (Portugal) and December 10 (Spain), 2002, batches of 5.0±0.25 g of alder or oak leaves were weighed, rehydrated, enclosed into tetrahedral coarse mesh bags (10 x 15 cm, 10 mm mesh) and deployed in streams the following day. A flood in

Table 1. Location, geology and physico-chemical characteristics (mean and range) of deciduous and eucalyptus streams in Spain and Portugal. *n*, number of measurements.

	Spain			Portugal		
	n	**Deciduous**	**Eucalyptus**	***n***	**Deciduous**	**Eucalyptus**
Latitude, N		43º19'30"	43º19'52"		40º4'44"	40º30'57"
Longitude, W		3º20'05"	3º19'37"		8º12'30"	8º18'23"
Altitude (m a.s.l.)		135	75		640	215
Catchment area (km^2)		4.1	5.7		0.3	2.0
Distance to source (km)		4.0	4.8		0.7	1.5
Geology		siliceous	siliceous		siliceous	siliceous
Dominant substrate type (cm)		20–40	6–40		20–40	6–20
Temperature (ºC)	1248	8.8 (2.4–13.2)	8.7 (2.9–15.8)	2278	9.2 (5.4–11.5)	11.7 (8.7–14.1)
Conductivity ($\mu S\ cm^{-1}$)	9	84 (54–113)	83 (54–116)	5	23 (22–23)	34 (32–38)
Alkalinity (mg $CaCO_3\ L^{-1}$)	9	25.1 (15.0–34.5)	22.8 (10.0–32.0)	3	4.5 (4.1–5.1)	4.4 (4.1–4.7)
NO_3-N ($\mu g\ L^{-1}$)	9	461.5 (198.0–652.0)	425.6 (237.0–570.0)	7	110.1 (79.5–153.8)	137.0 (98.3–204.4)
NH_4-N ($\mu g\ L^{-1}$)	9	42.2 (3.0–142.0)	52.5 (6.0–139.0)	4	<10.0–14.0	<10.0
SRP ($\mu g\ L^{-1}$)	9	9.4 (2.0–23.0)	<1.0–49.0	7	<2.0–7.0	3.4 (2.0–4.0)
pH	9	7.3 (6.6–7.6)	7.2 (6.9–7.5)	4	6.4 (6.3–6.6)	6.3 (6.2–6.5)

early January 2003 washed away most oak bags in Portugal, so new batches were prepared and deployed in the same streams on January 26, 2003, and the results from these bags will be presented here. A flood was also registered in Spain on February 4, 2003, affecting only oak bags (alder experiment was already finished). This flood, however, had less dramatic effects than that in Portugal allowing for two samplings after the event.

Leaf bags (22 per leaf species per stream) were tied by nylon lines (2 per line) to iron rebars driven into the stream bed along a 150 m reach. Alder leaf bags were retrieved randomly after 7, 13, 20, 26 and 42 days of incubation in Portugal, and after 7, 23, 28, 31 and 43 days in Spain, whereas oak leaves were retrieved after 14, 26, 43, 57 and 74 days in Portugal, and 23, 41, 65 and 104 days in Spain (4–6 replicates of each substrate type each time). Extra sets of leaves were prepared to calculate corrections for leaching and air dry mass to ash free dry mass (AFDM) conversion factors. After retrieval, bags were placed in individual zip lock bags and transported in ice chests to the laboratory where they were processed within 24 h. In the laboratory, leaf material from each bag was rinsed with distilled water and 2 sets of 5 leaf disks were cut out with a cork borer (12 mm diameter, see below). The remaining material was oven dried at 105 ºC for 24 h and weighed. A subsample was then taken, weighed and ashed at 550 ºC for 4 h and reweighed to calculate % ash and AFDM.

Nitrogen and phosphorus in leaves

A subsample of leaf material was ground into a fine powder (Retsch ZN100 or Culatti DFH48 mills, 1 mm screen) and analyzed for N and P in the same laboratory. Nitrogen was determined using a Perkin Elmer Series II CHNS/O analyzer. Phosphorus was determined spectrophotometrically at 700 nm, after mixed acid digestion (15 min. at 325 ºC; ALLEN, 1989). Results were expressed as % N and % P of the remaining leaf litter AFDM.

Fungal biomass

One set of leaf disks was used for ergosterol determination (GESSNER & SCHMITT, 1996; GESSNER, 2005), which is a measure of fungal biomass. Leaf disks were frozen and later freeze-dried just before extraction. For ergosterol extraction, leaf disks were placed in tightly closed tubes with 10 mL of KOH/methanol in a water bath (80 ºC) for 30 min., with stirring. The extract was then purified by solid-phase extraction (Waters Sep-Pak Vac RC tC_{18} cartridges) as described by GESSNER (2005). Ergosterol was quantified by high performance liquid chromatography (HPLC) by measuring absorbance at 282 nm. The HPLC system (Dionex DX-120) was equipped with the reverse phase C_{18} column (Brownlee SPHERI-5RP-

18, Applied Biosystems) maintained at 33 ºC. The mobile phase was 100 % methanol and the flow rate was set at 1.5 mL min^{-1}. Ergosterol was converted into fungal biomass with a conversion factor of 5.5 µg ergosterol mg^{-1} fungal dry mass (GESSNER & CHAUVET, 1993). Ergosterol analyses were done in Portugal and results were expressed as mg fungal biomass g^{-1} AFDM.

Sporulation of aquatic hyphomycete

The 2nd set of leaf disks was used to induce sporulation by aquatic hyphomycetes (BÄRLOCHER, 2005). Disks were incubated in 100 mL Erlenmeyer flasks with 25 mL of filtered stream water (glass fiber filter, Millipore APFF) on a shaker (100 rpm) for 48 h at 10 ºC. The conidia suspensions were decanted into 50 mL centrifuge tubes, flasks rinsed twice, and conidia fixed with 2 mL of 37 % formalin to be later counted and identified. When preparing slides for conidia identification in Portugal 100 µL of Triton X-100 solution (0.5 %) were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot of the suspension was filtered (Millipore SMWP, 5 µm pore size). Filters were stained with cotton blue in lactic acid (0.05 %), and spores were identified and counted with a compound microscope at 200x. Leaf disks AFDM was determined as described above for bulk leaf material. Sporulation rates were expressed as number of conidia released mg^{-1} AFDM day^{-1}.

DATA ANALYSIS

After correcting leaf litter initial mass for leaching, decomposition rates, *k*, were calculated by linear regression of ln transformed data (negative exponential model $M_t = M_o \cdot e^{-kt}$, where M_o is the initial mass, M_t is the remaining mass at time t, and *k* is the decomposition rate). As there were differences in temperature between streams (**Table 1**), decomposition rates were expressed in terms of degree $days^{-1}$ by replacing time (t) by the sum of mean daily temperatures accumulated by the sampling day. Decomposition rates were compared by ANCOVA with country and stream type as categorical variables and time as continuous variable, followed by Tukey's test (ZAR, 1999).

Evenness (Pielou's index, J') of fungal communities from decomposing leaves was calculated from conidial abundances (PRIMER 6). Pielou's evenness index describes the distribution of individuals by species in a community and varies between 0 (few dominant species) and 1 (many codominant species) (WASHINGTON, 1984). Comparisons of N and P content of litter, fungal biomass and sporulation, aquatic hyphomycete species richness and

evenness of fungal communities associated with decomposing leaves between deciduous and eucalyptus streams, within each country, for each substrate were done by 2-way ANOVA with stream type and time as categorical variables. Comparisons between deciduous or eucalyptus streams between countries were done by t test of mean values over time (N and P content of leaves) or peak values (fungal biomass and sporulation, species richness and Pielou's index).

Multidimensional scaling (MDS) based on Bray-Curtis similarity matrix of relative abundances of aquatic hyphomycetes in both stream types (all samples considered) for each leaf species and country was performed with PRIMER 6. Differences in aquatic hyphomycete communities between stream types were assessed by analysis of similarities (ANOSIM, PRIMER 6; CLARKE & GORLEY, 2001). Statistical analyses were performed with STATISTICA 6 unless otherwise indicated.

RESULTS

Decomposition

Alder leaves decomposed fast in both countries with 28–54 % of AFDM remaining after 4–6 weeks (**Fig. 1**). Oak leaves decomposed more slowly with 58 % of AFDM remaining after 11 weeks of incubation in Portugal and 12–41 % after 15 weeks of incubation in Spain. Generally, mass loss patterns were similar in both countries, and no significant differences in decomposition rates were found between stream types or between countries for either alder or oak (ANCOVA, $p>0.05$). The only exception was oak leaves in the eucalyptus stream in Spain, which decomposed faster than in the deciduous stream or in the eucalyptus stream in Portugal (ANCOVA, $p<0.001$; **Table 2**).

Nitrogen and phosphorus in leaves

Nitrogen content of leaves generally increased through time in both alder and oak (**Fig. 2**), suggesting microbial N immobilization. Leaves incubated in the eucalyptus stream had significantly higher N content than leaves in the deciduous stream in both countries (2-way ANOVA, $p<0.038$), except for alder leaves in Spain (2-way ANOVA, $p=0.749$). Nitrogen content of alder leaves incubated in the deciduous stream was higher in Spain than in Portugal (t test, $p=0.013$).

The dynamics of phosphorus in leaves was not consistent between streams or countries (**Fig. 3**). P content of alder leaves decreased (Spain), remained constant (Portugal, deciduous stream) or increased (Portugal, eucalyptus stream) through time, being significantly higher in

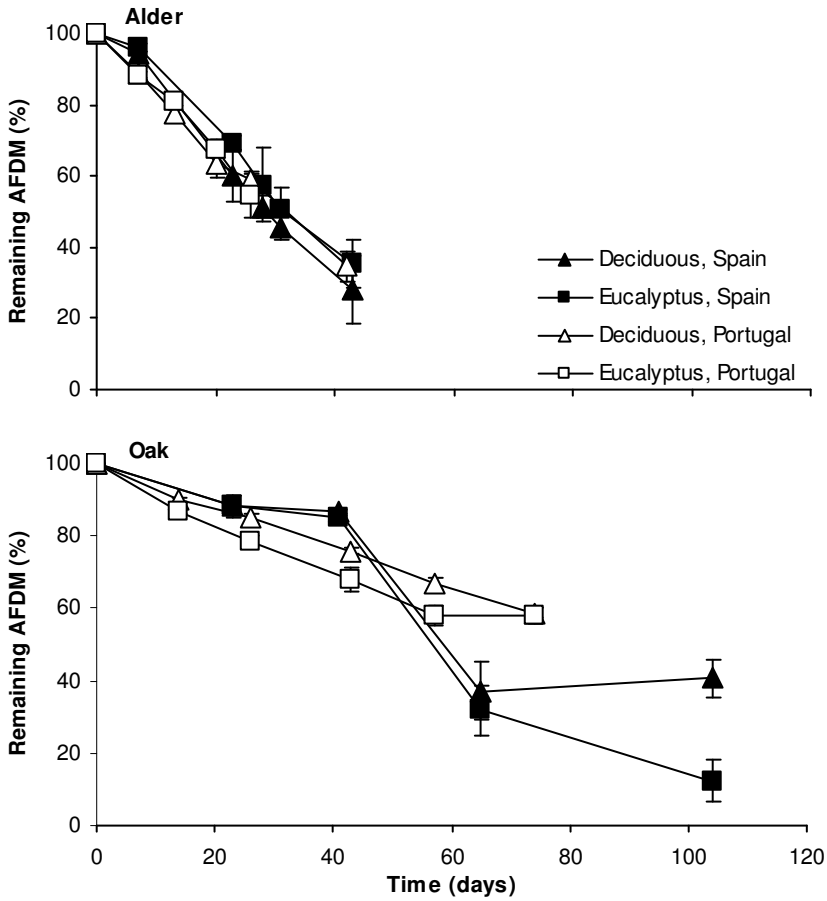

Figure 1. Remaining mass (mean±1SE) of alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

Table 2. Decomposition rates *k* (degree-day^{-1}, 95 % CL in parenthesis) of alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal. For each leaf species, decomposition rates with the same letter were not significantly different (ANCOVA followed by Tukey's test, p>0.050).

		Alder		Oak	
Spain	**Deciduous**	0.0032 (0.0004)	a	0.0012 (0.0002)	a
	Eucalyptus	0.0024 (0.0002)	a	0.0025 (0.0003)	b
Portugal	**Deciduous**	0.0024 (0.0001)	a	0.0008 (<0.0001)	a
	Eucalyptus	0.0019 (0.0002)	a	0.0008 (<0.0001)	a

R^2 varied between 0.53 and 0.97

the eucalyptus than in the deciduous stream in Portugal (2-way ANOVA, p=0.012). Oak leaves in Portugal had 6 times higher initial P content than in Spain, and after the initial decrease, P content remained relatively constant until the end of the experiment, while in Spain it remained constant throughout the experiment. In both countries, P content of oak leaves was significantly higher in the eucalyptus than in the deciduous stream (2-way

ANOVA, p<0.018). Phosphorus content of leaves was higher in Portugal than in Spain (t test, p<0.005), except for alder leaves in the deciduous streams where there were no significant differences (t test, p=0.403).

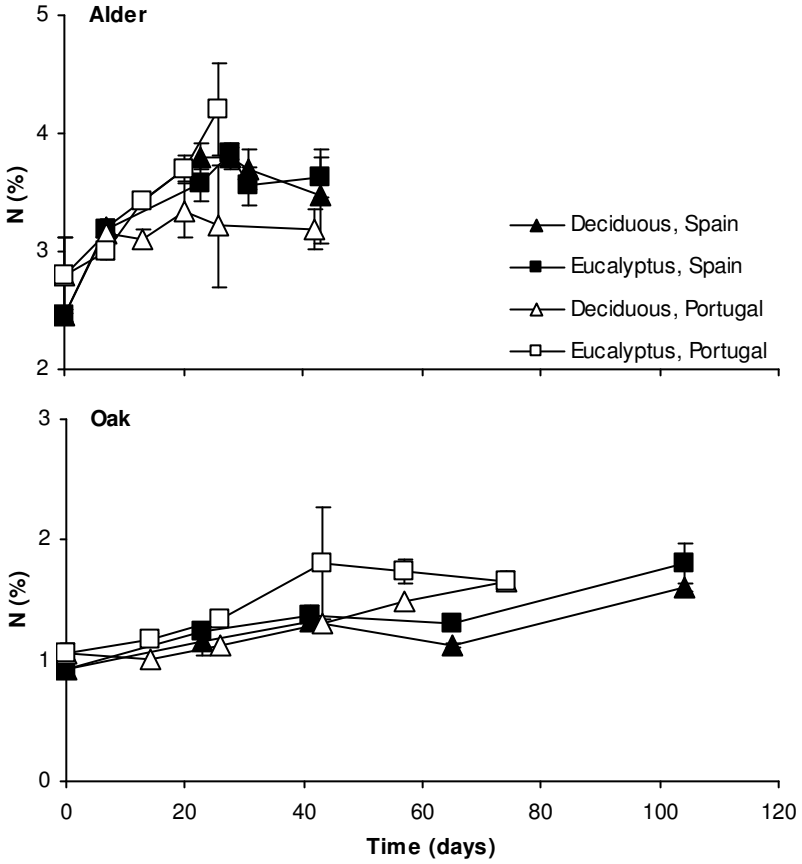

Figure 2. Nitrogen content (mean±1SE) of alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

Fungal biomass

Fungal biomass associated with alder leaves peaked earlier in the deciduous than in the eucalyptus stream, in both countries (day 14 *vs*. 20 in Portugal and day 23 *vs*. 28 in Spain; **Fig. 4**), however, eucalyptus streams had significantly higher fungal biomass than deciduous ones (2-way ANOVA, p<0.017). Although the fungal biomass peaked earlier in Portugal than in Spain, no significant differences were found in peak values between countries (t test, p>0.099).

Fungal biomass associated with oak leaves in eucalyptus streams peaked by days 41–57 while in deciduous streams it increased through time (**Fig. 4**). Leaves incubated in the

eucalyptus stream had higher fungal biomass than leaves in the deciduous stream in both countries (2-way ANOVA, $p<0.001$). Maximum fungal biomass associated with decomposing oak was higher in Portugal than in Spain (t test, $p<0.014$).

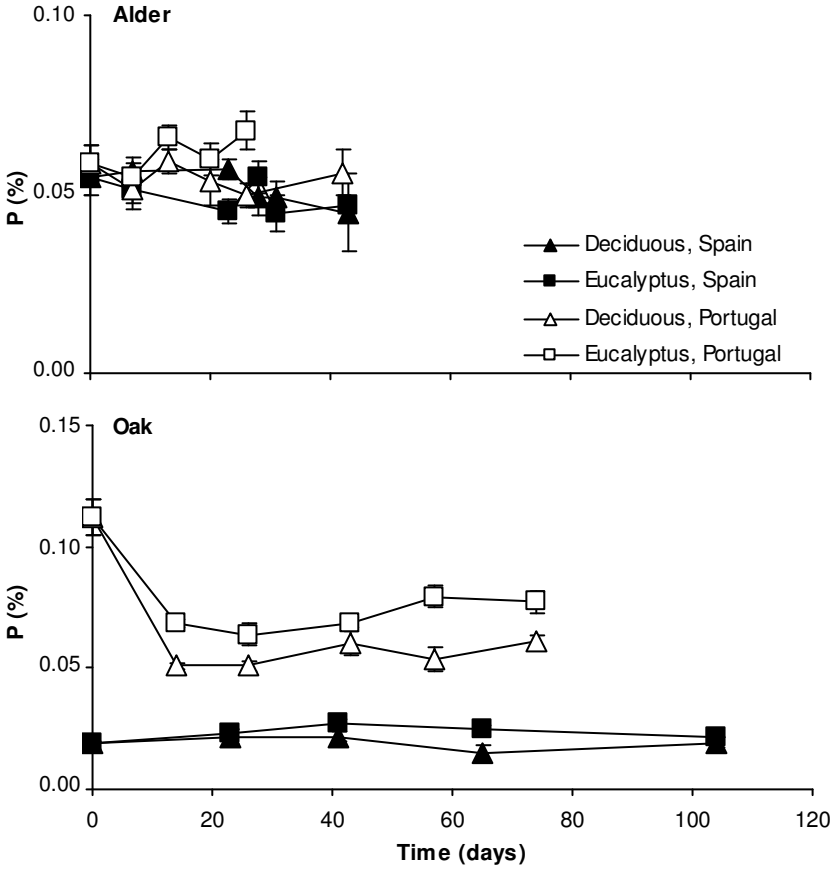

Figure 3. Phosphorus content (mean±1SE) of alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

Sporulation of aquatic hyphomycetes

Maximum sporulation rates of aquatic hyphomycetes associated with alder leaves tended to be higher in eucalyptus than in deciduous streams (2288 *vs*. 1128 conidia mg^{-1} AFDM d^{-1} in Spain and 2969 *vs*. 1311 conidia mg^{-1} AFDM d^{-1} in Portugal; **Fig. 5**). However, no overall significant differences were found between stream types in both countries (2-way ANOVA, $p>0.239$). No significant differences were found in the peak values between streams in Portugal and Spain (t test, $p>0.594$).

Maximum sporulation rates associated with oak leaves were higher in eucalyptus than in deciduous streams (828 *vs*. 492 conidia mg^{-1} AFDM d^{-1} in Portugal and 141 *vs*. 19 conidia

mg^{-1} AFDM d^{-1} in Spain; **Fig. 5**). Sporulation in the Spanish eucalyptus stream was significantly higher than in the deciduous stream (2-way ANOVA, $p<0.001$). Streams in Portugal had significantly higher maximum sporulation rates than streams in Spain (t test, $p<0.009$).

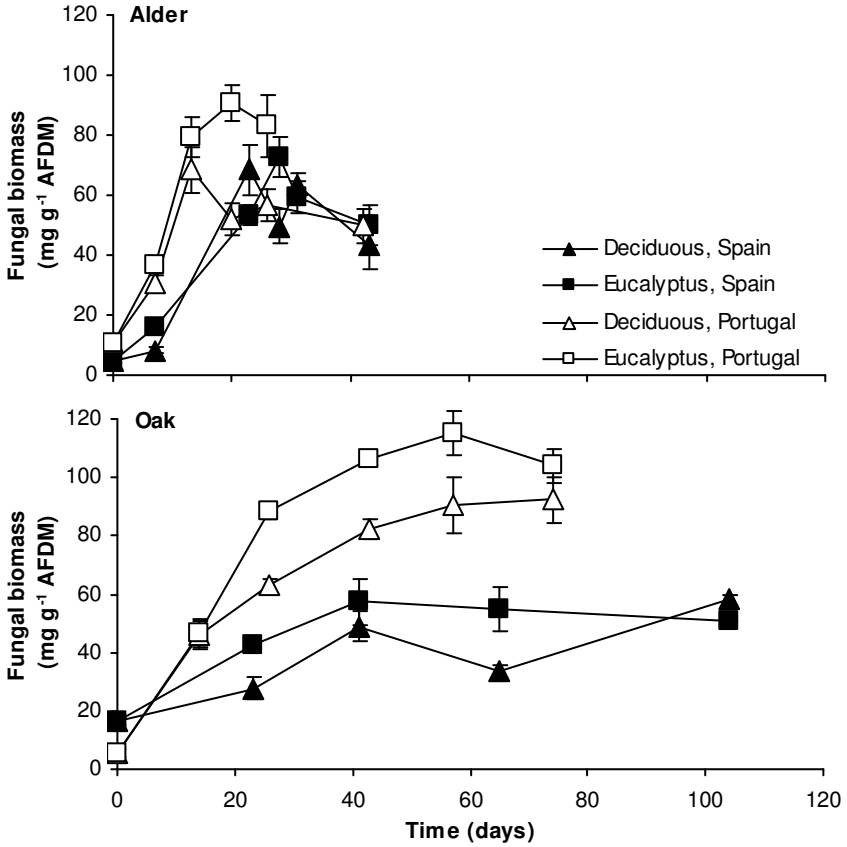

Figure 4. Fungal biomass (mean±1SE) associated with alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

Aquatic hyphomycete communities

Thirty three species of aquatic hyphomycetes have been found in this study, 23 in Portugal and 29 in Spain (**Table 3**). Species richness was higher in the eucalyptus than in the deciduous stream in Spain (21 *vs*. 12 species on alder and 24 *vs*. 19 on oak) while in Portugal it was the opposite (14 *vs*. 17 on alder and 16 *vs*. 20 on oak). *Flagellospora curvula* dominated the fungal communities on both leaf species in both streams in Spain (mean relative abundance 82.9–89.2 % on alder and 44.1–53.6 % on oak). *F. curvula* was an early colonizer with its maximum spore production during the first weeks of litter decomposition that

coincided with the overall sporulation peak. As its abundance declined during oak litter decomposition, *Lunulospora curvula* (7.3–19.4 %) and *Articulospora tetracladia* (8.7–14.9 %) became codominant while on alder the relative abundance of *F. curvula* never declined below 66 %. *Tetrachaetum elegans* dominated on both alder and oak leaves in the deciduous stream in Portugal (32.9–36.6 %) while *Anguillospora filiformis* (51.3 %) and *L. curvula* (29.6 %) dominated on alder leaves and *Tricladium chaetocladium* (48.7 %) and *A. filiformis* (38.5 %) on oak leaves in the eucalyptus stream.

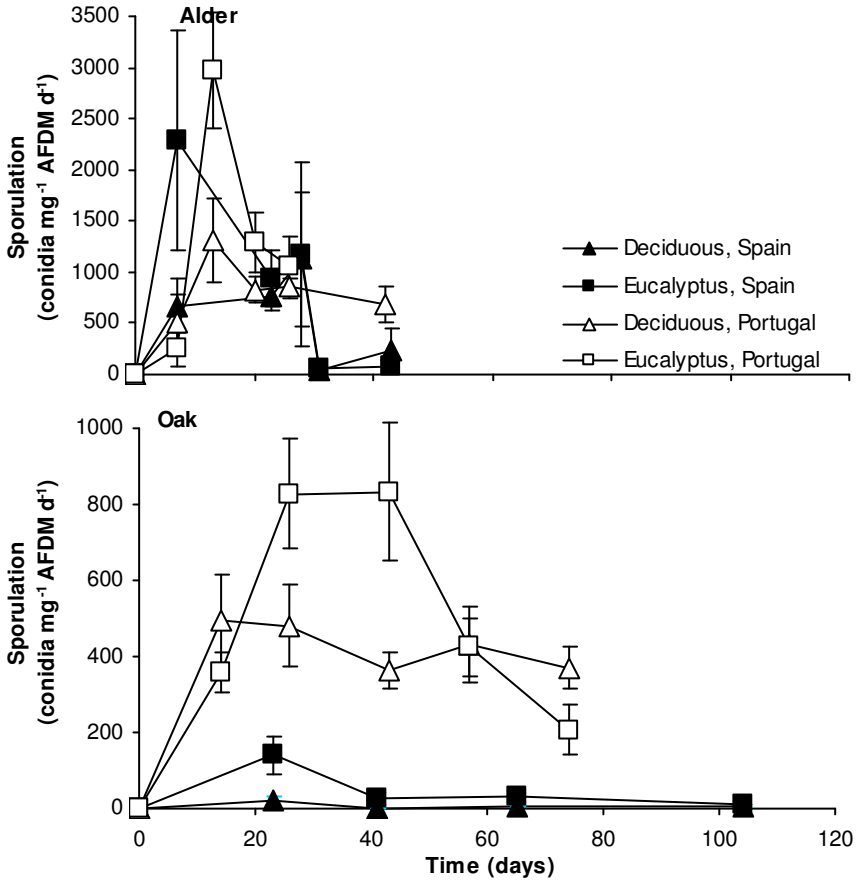

Figure 5. Sporulation rate (mean±1SE) of aquatic hyphomycetes associated with alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

Although there was a tendency for leaves incubated in Portugal to have higher fungal species richness and evenness in the deciduous than in the eucalyptus stream (**Fig. 6**; **Table 3**) differences between stream types were significant only for the Pielou's index for alder and species richness for oak leaves (2-way ANOVA, $p<0.028$). Species richness for both leaf species and Pielou's index for alder were higher in the eucalyptus than in the deciduous stream

in Spain (2-way ANOVA, $p<0.019$). Alder leaves incubated in the deciduous stream in Portugal had higher maximum number of species and evenness than leaves incubated in Spain, (t test, $p=0.003$ and 0.001, respectively), while the opposite was true for the maximum number of species for alder and oak leaves in the eucalyptus stream (t test, $p=0.031$ and 0.006, respectively).

Table 3. Mean relative abundances (%, over all sampling dates) of aquatic hyphomycete conidia from alder and oak leaves incubated in deciduous (D) and eucalyptus (E) streams in Spain and Portugal. Total number of species and mean evenness indices are also given. +, mean relative abundance <0.1%.

	Alder				Oak			
	Spain		Portugal		Spain		Portugal	
Aquatic hyphomycete species	**D**	**E**	**D**	**E**	**D**	**E**	**D**	**E**
Alatospora acuminata INGOLD s.l.	0.8	1.1	1.5	0.5	6.3	5.1	2.2	0.2
Alatospora flagellata (J. GÖNCZÖL) MARVANOVÁ			+		0.2	+	0.1	
Alatospora pulchella MARVANOVÁ			0.1	+	0.1		4.0	0.3
Anguillospora crassa INGOLD						0.3		
Anguillospora filiformis GREATH.	0.5	0.9	9.2	51.3	0.5	1.1	10.7	38.5
Anguillospora cf. *furtiva* J. WEBSTER & DESCALS						+		
Anguillospora cf. *longissima* (SACC. & P. SYD.) INGOLD		0.1						
Articulospora tetracladia INGOLD	6.0	4.9	1.7	1.1	14.9	8.7	1.9	1.2
? *Calcarispora hiemalis* MARVANOVÁ & MARVAN		+			0.1	0.3		
Clavariopsis aquatica DE WILD.	0.1	0.3			2.3	2.9	+	
Clavatospora longibrachiata (INGOLD) MARVANOVÁ & SV. NILSSON	0.1	1.4	8.3	0.5	1.9	2.3	11.1	2.0
Crucella subtilis MARVANOVÁ & SUBERKR.		+	11.0			0.1	2.3	
Flagellospora curvula INGOLD	89.2	82.9	14.4	0.1	53.6	44.1	4.2	0.4
Goniopila monticola (DYKO) MARVANOVÁ & DESCALS		+	0.5			0.2	0.1	
Heliscella stellata (INGOLD & V.J. COX) MARVANOVÁ		0.3	3.4	+	4.7	1.5	8.3	0.1
Heliscus lugdunensis SACC. & THERRY		0.1						
Lemonniera aquatica DE WILD.				0.7			+	0.3
Lemonniera terrestris TUBAKI								0.1
Lunulospora curvula INGOLD	1.4	4.4		29.6	7.3	19.4		5.9
Stenocladiella neglecta (MARVANOVÁ & DESCALS) MARVANOVÁ & DESCALS	+	0.4	0.8		+	2.1	0.3	
Taeniospora gracilis var. *enecta* MARVANOVÁ & STALPERS			0.3			0.1	0.4	
Tetrachaetum elegans INGOLD	1.4	2.6	32.9	0.4	4.7	3.3	36.6	0.5
Tetracladium marchalianum DE WILD.	+	+						
Tricladium chaetocladium INGOLD		0.3	1.4	12.0		2.9	16.3	48.7
Tricladium splendens INGOLD							+	
Triscelophorus acuminatus NAWAWI		+	8.9	0.9		0.3	0.1	0.3
Triscelophorus monosporus INGOLD		+			1.0	4.0		
Variocladium giganteum (S.H. IQBAL) DESCALS & MARVANOVÁ					1.2	0.2		
Sigmoid conidia (<60 µm long)	0.1	0.1	5.5	2.7	0.6	0.1	1.3	1.4
Sigmoid conidia (60–120 µm long)					0.2			
Sigmoid conidia (>120 µm long)					0.1	0.1		+
Unidentified tetraradiate conidia	0.3	0.1	0.1	0.2	0.2	0.9	0.2	0.1
Total no. species	12	21	17	14	19	24	20	16
Pielou's evenness index, J'	0.28	0.33	0.70	0.59	0.65	0.60	0.62	0.57

Fungal communities colonizing alder and oak leaves in Spain were different between the deciduous and the eucalyptus stream although there was some overlap (ANOSIM, p=0.001 and R=0.52 for alder and p=0.002 and R=0.33 for oak; **Fig. 7**) while in Portugal both stream types were completely separated based on their fungal communities (ANOSIM, p=0.001 and R=0.92 for alder and p=0.001 and R=1.00 for oak; **Fig. 7**).

DISCUSSION

Using identical protocols, this study assessed the effect of eucalyptus plantations on fungi associated with leaf litter decomposition in Portuguese and Spanish streams. The 2 streams in each country were selected to be similar with respect to geology, morphology, substrate and physico-chemical parameters and to differ only in riparian vegetation. However, there were some differences in water temperature and initial P content of oak litter between countries that may have confounded some comparisons. It is still difficult to generalize about the impact of eucalyptus plantations on aquatic fungal communities and associated processes since the eucalyptus stream in Portugal was apparently more affected than that in Spain. This differential effect of eucalyptus plantations probably resulted from differences in climate between the two regions.

In agreement with previous studies using the same and other litter types (e.g. MOLINERO *et al.*, 1996; POZO *et al.*, 1998; DÍEZ *et al.*, 2002; BÄRLOCHER & GRAÇA, 2002), breakdown rates of alder and oak leaves in Portugal did not differ significantly between eucalyptus and deciduous forest streams. In Spain, however, oak leaves decomposed significantly faster in the eucalyptus than in the corresponding deciduous stream and than in the eucalyptus stream in Portugal. These differences were most likely brought about by physical abrasion caused by the flood in early February in Spain. Before this event breakdown dynamics of oak leaves was very similar in both the deciduous and the eucalyptus stream.

Litter nutrient dynamics has been reported to be closely associated with microbial dynamics (e.g. SAMPAIO *et al*. 2001; GULIS & SUBERKROPP 2003) as fungal biomass is nutrient rich. Nitrogen content of both leaf types increased through time as expected following fungal colonization (e.g. MOLINERO *et al.*, 1996; POZO *et al.*, 1998; SAMPAIO *et al.*, 2001; GULIS & SUBERKROPP, 2003) while phosphorus showed a more erratic pattern. The 6 times higher initial P content of oak leaves in Portugal than in Spain was a surprise (MOLINERO *et al.*, 1996; SAMPAIO *et al.*, 2001). Generally there was higher nutrient content in leaves incubated in eucalyptus than in deciduous streams, which could be a result of higher fungal

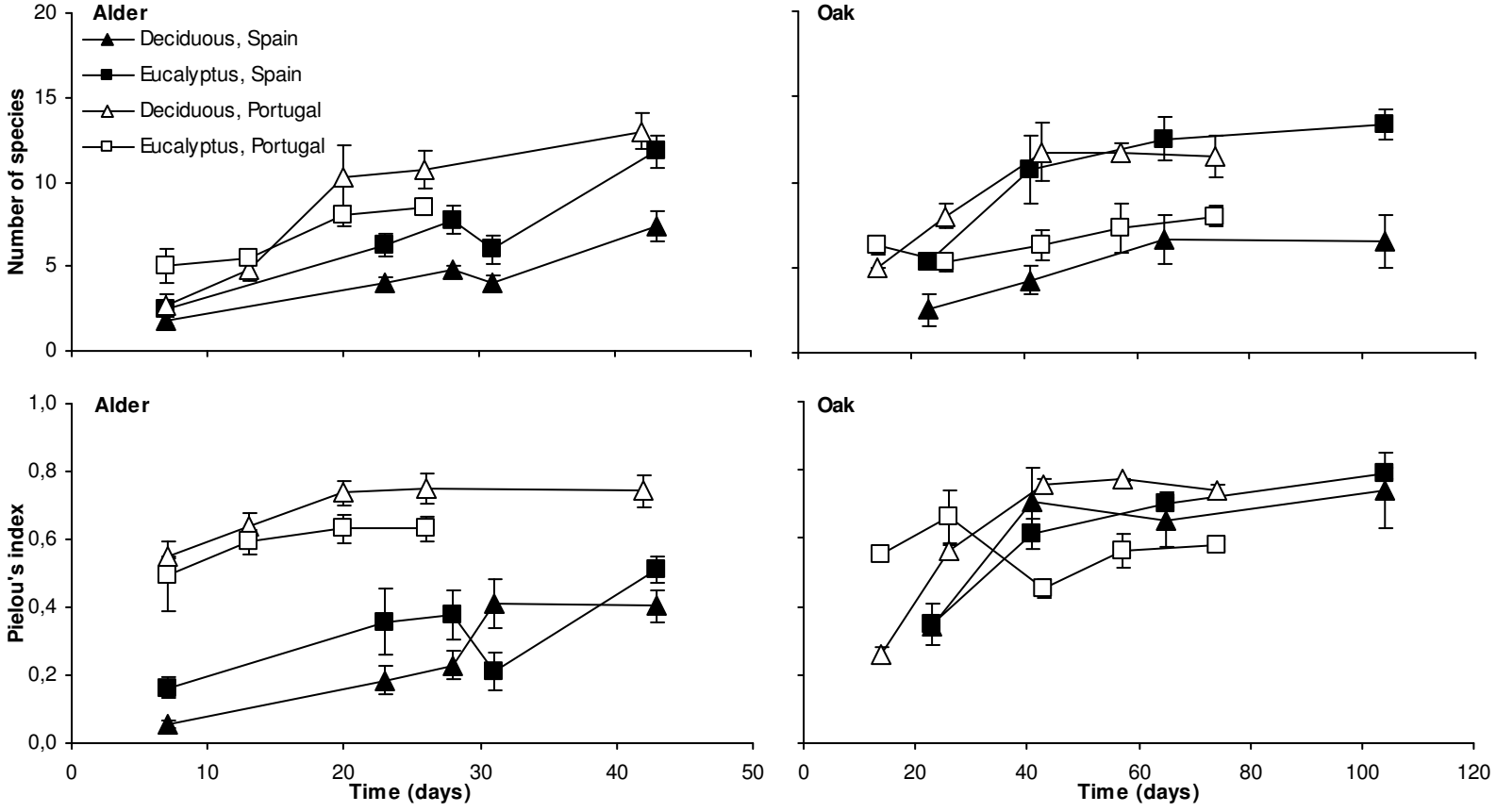

Figure 6. Species richness of aquatic hyphomycetes (mean±1SE) and Pielou's evenness of fungal communities (mean±1SE) associated with alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal.

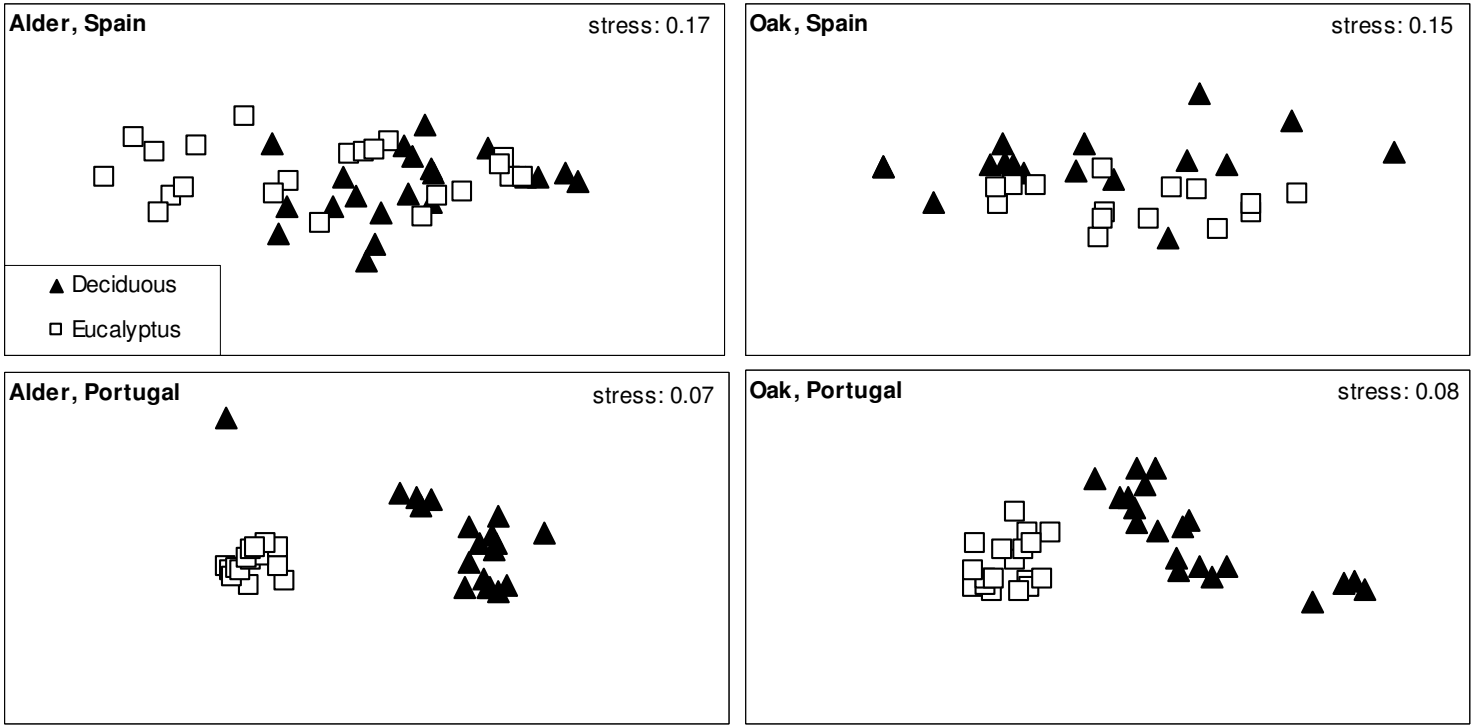

Figure 7. MDS ordination of fungal assemblages colonizing alder and oak leaves incubated in deciduous and eucalyptus streams in Spain and Portugal. The stress was <0.20, the 2D ordination plot therefore faithfully represented the multi-dimensional relationships among the samples.

activity in the former ones (see below). Our findings contradict some previous reports. MOLINERO *et al.* (1996) found no significant differences in the nutrient contents of oak, chestnut and eucalyptus leaves between stream types and POZO *et al.* (1998) found higher N content in alder leaves incubated in a deciduous than in those incubated in a eucalyptus stream. Nutrient content was also higher in Portuguese than in Spanish streams, probably as a consequence of higher fungal activity in Portuguese streams.

Fungal biomass and sporulation rates were generally higher in leaves incubated in eucalyptus than in leaves incubated in deciduous streams in both countries. In contrast, CHAUVET *et al*. (1997) and POZO *et al.* (1998) found no significant differences in these parameters between stream types. In this study, differences in fungal activity between stream types might have resulted not only from differences in the forest cover but also in stream water temperature. Higher water temperatures are known to stimulate the activity of some fungal species (CHAUVET & SUBERKROPP, 1998; GRAÇA & FERREIRA, 1995), and eucalyptus streams had higher water temperatures than deciduous streams. Higher temperatures in Portuguese than in Spanish streams could also explain higher sporulation rates and fungal biomass in the former ones, in spite of higher nutrient content in Spanish streams. The higher fungal activity in eucalyptus streams might also be a result of differences in litter dynamics. Although eucalyptus streams receive lower litter inputs, their benthic storage is higher than in deciduous streams as a result of the summer peak in litter input that coincides with a period of low discharge (POZO *et al.*, 1997a, b; MOLINERO & POZO, 2003, 2004). Since aquatic hyphomycetes are sensitive to changes in the amount of CPOM in stream (LAITUNG *et al.*, 2002), streams with higher benthic storage might be expected to support higher fungal activity.

Although fungal activity was generally higher in the eucalyptus than in the deciduous stream in Portugal, the species richness and evenness of fungal communities were higher in the deciduous stream, which agrees with the previous report by BÄRLOCHER & GRAÇA (2002). This suggests a certain level of functional redundancy among species of aquatic hyphomycetes when loss of some species does not translate into appreciable decrease in ecosystem functioning (decomposition) (WALKER, 1992; DANG *et al.*, 2005). However, in Spain, we found higher species richness and higher evenness of fungal communities in the eucalyptus than in the deciduous stream, which is difficult to explain unless the understory of young deciduous trees in the eucalyptus stream had a major effect (see below).

Stream types in Portugal were completely separated based on their fungal communities (MDS analysis) where 7 species were found only in the deciduous stream. Overlap in fungal communities was greater in Spain, even though 9 rare species were restricted to the eucalyptus stream. This suggests that the effect of eucalyptus plantation on fungal community structure was probably stronger in Portugal than in Spain. There are several possible explanations. (a) The eucalyptus plantation in Spain had young deciduous trees in the understory that increased the diversity of leaf litter entering the eucalyptus stream. This may support higher fungal diversity than in the eucalyptus stream in Portugal, which was bordered almost exclusively by eucalyptus trees. (b) Precipitation in northern Spain is higher and more evenly distributed throughout the year. In central Portugal, precipitation is highly seasonal with summer droughts affecting stream flow, benthic organic matter accumulation, water temperature and chemistry. All these effects are more pronounced in eucalyptus streams. Eucalyptus plantations have strongly hydrophobic soils, which have lower infiltration rates resulting in higher runoff in winter and lower stream water level in summer (streams can be reduced to ponds or completely dry out) (ABELHO & GRAÇA 1996). The tree canopy of eucalyptus plantations is more open than in deciduous forests because trees are generally young and regularly spaced, which allows for a greater water surface to be exposed to solar radiation (MOLINERO & POZO 2003). Reduced flow and higher water temperature (higher evaporation) results in increased ionic strength and concentration of phenolic compounds and decreased dissolved oxygen in eucalyptus streams.

Fungal communities colonizing alder and oak leaves were quite different between the two countries. *Flagellospora curvula* dominated fungal assemblages on both leaf species in both streams in Spain with *Lunulospora curvula* and *Articulospora tetracladia* as codominants. The dominance of *F. curvula* and *L. curvula* in Spanish streams was also reported by CHAUVET *et al*. (1997). *Tetrachaetum elegans*, *Anguillospora filiformis*, *Tricladium chaetocladium* and *L. curvula* were the most abundant species in Portugal. As observed before (BÄRLOCHER *et al*., 1995; CHAUVET *et al*., 1997; BÄRLOCHER & GRAÇA, 2002; PASCOAL *et al*., 2005), *F. curvula* did not attain the same abundances in Portugal as it did in Spain (0.1–14.4 % *vs*. 44.1–89.2 %), which could be explained by the higher water temperatures in Portuguese streams and the preference of this species for colder waters (CHAUVET & SUBERKROPP, 1998). Also, *L. curvula* was more abundant in Portuguese than in Spanish streams as previously reported (BÄRLOCHER *et al*., 1995; CHAUVET *et al*., 1997; BÄRLOCHER & GRAÇA, 2002), which could again be explained by higher water temperature in

Portuguese streams. The water temperature during the study period and across streams varied between 2.4 and 15.8 ºC. The importance of *L. curvula* in this study therefore contrasts with previous reports that associated this species with warm waters (reviewed by BÄRLOCHER, 1992). In a summer cool stream (<15 ºC), GESSNER *et al*. (1993) found a rather similar fungal community although species like *L. curvula* and *Triscelophorus monosporus*, considered warm water species, were not present.

The substitution of native forests by exotic monocultures has been associated with changes in sporulation dynamics of aquatic hyphomycetes (THOMAS *et al*., 1989) and decreases in fungal diversity and species richness (BÄRLOCHER & GRAÇA, 2002). LAITUNG & CHAUVET (2005) also reported a strong effect of leaf litter abundance and diversity on conidia concentration in water, fungal species richness and diversity. However, in studies where leaf litter decomposition was simultaneously assessed, changes in fungal communities did not result in altered litter breakdown (BÄRLOCHER & GRAÇA, 2002; this study). Changes in aquatic hyphomycete diversity but not in litter decomposition were also observed in 2 sites differing in water chemistry (PASCOAL *et al*., 2005) and in microcosm experiments (DUARTE *et al*., 2006). This could be explained by extensive functional redundancy among species of aquatic hyphomycetes (DANG *et al*. 2005), although BÄRLOCHER & CORKUM (2003) found that both species diversity and identity had significant effects on mass loss.

Nevertheless, as shredders can discriminate among leaf patches colonized by different fungal species (reviewed by SUBERKROPP, 1992), changes in fungal diversity can indirectly affect litter decomposition in streams where shredders are abundant and diverse. This was not the case in our study where the abundance and species richness of shredders was low.

Two main conclusions can be drawn from this study. First, stream fungal communities were more affected by riparian *Eucalyptus globulus* plantations in Portugal than in Spain. This is probably due to climatic differences. More humid conditions in Spain may facilitate survival of deciduous trees in the understory of eucalyptus plantations and, thus, litter inputs to eucalyptus streams may be less impoverished than in Portugal. Although there can be differences between stands, POZO *et al*. (1997a, b) reported that 2 % of leaves under eucalyptus plantations were deciduous and BAÑUELOS *et al*. (2004) found 8 % of leaf material to be oak. Second, diversity and activity of aquatic hyphomycetes were more affected by eucalyptus plantations than leaf litter decomposition, probably as a result of functional redundancy among fungal species.

To mitigate the effect of eucalyptus plantations on aquatic communities, deciduous trees could be planted on the river banks or, preferably, riparian buffer strips of native vegetation should be kept. These would increase the diversity of litter input to streams and, hence, allow higher fungal diversity. Also, native buffer strips can regulate air and water temperature by reducing the amplitude of daily variations (MELEASON & QUINN, 2004) as they may develop a closed canopy above small streams. The presence of these riparian buffer strips is also of major importance when the plantations are harvested as they continue to provide shading and CPOM input to streams (BOOTHROY *et al*., 2004; QUINN et al. 2004) preventing the shift from heterotrophy to autotrophy and associated decreases in fungal diversity and abundance. The importance of native riparian buffer strips in alleviating the effects of eucalyptus plantations on aquatic ecosystems has also been emphasized in previous studies (POZO *et al*., 1997b; MOLINERO & POZO, 2003, 2004).

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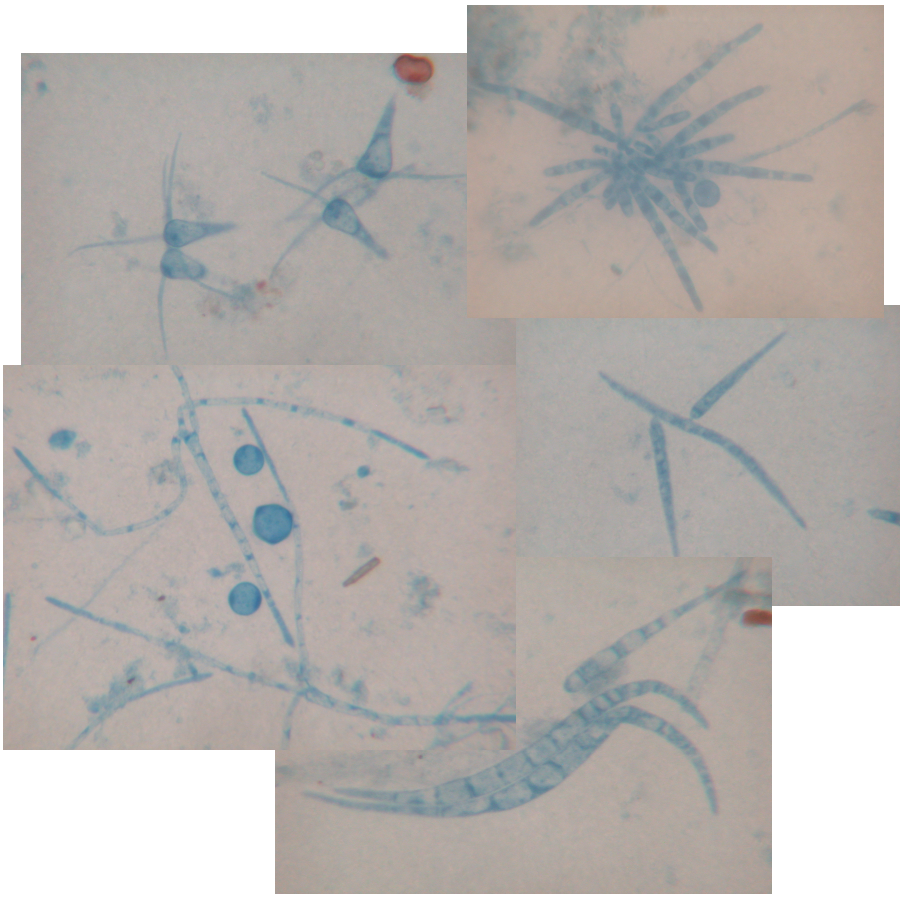
CHAPTER V

FUNGAL COMMUNITIES STRUCTURE IN LEAVES IS AFFECTED BY CURRENT VELOCITY BUT NOT BY INVERTEBRATE ACTIVITY



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Photos:

Clavariopsis aquatica

Dendrospora erecta

Tetrachaetum elegans and *Magaritispora aquatica*

Tricladium splendens

Anguillospora crassa

FUNGAL COMMUNITIES STRUCTURE IN LEAVES IS AFFECTED BY CURRENT VELOCITY BUT NOT BY INVERTEBRATE ACTIVITY

ABSTRACT

In this study it was evaluated the effect of current velocity and shredder presence, manipulated in artificial channels, in the structure of the fungal communities colonizing alder (*Alnus glutinosa* (L.) GAERTNER) leaves incubated in coarse and fine mesh bags. Fungal sporulation rates, cumulative conidial production and number of species of aquatic hyphomycetes were higher in leaves exposed to high rather than low current velocity. The opposite was observed regarding Simpson's index (D) on the fungal communities. Some species of aquatic hyphomycetes were consistently stimulated in high current channels. No effect of shredder or mesh type was observed. Thus, in studies where the aquatic fungal community structure is to be addressed careful should be taken regarding the current conditions where the litter is going to be emerged.

Key-words: alder leaves, aquatic hyphomycetes, artificial channels, *Seriscostoma* sp., shredders

INTRODUCTION

In forested low order streams, the primary energy source for aquatic communities is allochthonous organic matter provided by trees in the riparian zone (VANNOTE *et al*., 1980), mostly in the form of leaves (POZO *et al.*, 1997). Decomposition is a vital ecological process, mostly carried out by fungi and invertebrate decomposers (GESSNER *et al*., 1999; HIEBER & GESSNER, 2002). Fungal activity causes leaf decomposition, and appreciable amount of detrital carbon is converted into mycelial and conidial biomass (GESSNER & CHAUVET, 1994). Invertebrates, in particular leaf-shredding invertebrates, have also been shown to be important decomposers (e.g. CUFFNEY *et al*., 1990; STEWART, 1992). However, shredders seem to depend strongly on microbial colonization of leaves (GRAÇA *et al*., 2001). Shredders may benefit from fungal colonization because the accumulation of fungal biomass enhances the litter quality (conditioning) as (1) fungal enzymes aid in digestion of leaf tissues and (2) fungi are food for shredders (BÄRLOCHER & KENDRICK, 1975; ARSUFFI & SUBERKROPP, 1988). Shredders can discriminate between leaf patches colonized by different fungal species and at different degrees of conditioning (ARSUFFI & SUBERKROPP, 1985, 1986, 1989). Thus, by feeding selectively on certain fungal species or in leaf areas rich in fungal cells, shredders might directly influence the fungal community structure. This can be assessed by comparing decomposing leaves (a) between bags protected and unprotected from shredders or (b) between streams with (natural condition) and without invertebrates (by excluding them with insecticide) (SUBERKROPP, 2003).

However, results generated so far are inconclusive. Using the first approach, BÄRLOCHER observed that feeding activity by invertebrates lowered fungal species richness (BÄRLOCHER, 1980) or fungal sporulation rates (BÄRLOCHER, 1982) in oak leaves and larch needles incubated in coarse mesh bags, when compared with fine mesh bags. This was attributed to shredders likely acting as competitors through promoting fast mass loss in coarse mesh bags, thus reducing the space available for late colonizer species (BÄRLOCHER, 1980) or to the direct consumption of early colonizing aquatic hyphomycetes by the shredder *Gammarus fossarum* (BÄRLOCHER, 1982). On the other hand, ROSSI (1985) found higher diversity of aquatic fungi, and SABETTA *et al*. (2000) found higher respiration and number of species of aquatic fungi in leaves unprotected from invertebrates than in leaves protected from invertebrates. This could be explained by damage of the leaf epidermis by the invertebrate activities and thus facilitating fungal colonization and establishment (SUBERKROPP & KLUG, 1976). GRAÇA *et al*. (2002) also found that invertebrate shredding can stimulate fungal growth

on decaying litter, when grazing intensity is not high. Then again, HOWE & SUBERKROPP (1994) found no significant differences in fungal species richness, microbial respiration or biomass in poplar leaves protected and unprotected from the consumer *Lirceus* sp. Only sporulation rates were significantly different between bag types, but this was attributed to a reduction in water flow in fine mesh bags. Using the second approach, SUBERKROPP & WALLACE (1992) compared the fungal community in leaves incubated in an insecticide treated stream and two untreated reference streams and found no significant differences in fungal species composition between treated and untreated streams, although in the treated stream fungi sporulated at higher frequencies.

Current velocity can also be an important factor regulating the fungal community structure. It has been shown that aquatic hyphomycetes colonize preferentially oxygen rich waters (CHAUVET, 1992) and turbulence stimulates spore release. Thus, the use of fine mesh bags to assess the fungal community structure can be compromised since fine mesh might unnaturally reduce physical leaf fragmentation and change water flow patterns (SUBERKROPP, 2003). This may in turn lead to a reduction in species diversity and/or sporulation rates (HOWE & SUBERKROPP, 1994).

In this study it was assessed the effect of current velocity and shredder presence, manipulated in artificial channels, in the structure of the fungal communities colonizing alder (*Alnus glutinosa* (L.) GAERTNER) leaves incubated in coarse and fine mesh bags. Manipulation allowed us to compare fungal community structure and sporulation rates (1) between coarse and fine mesh bags, assessing in this way the effect of the mesh size, and (2) between coarse mesh bags placed in channels differing in current velocity and invertebrate density, assessing in this way the effect of both current velocity and invertebrate activity. It was expected fungal sporulation rates to be higher in leaves incubated in coarse mesh bags at higher current velocity and in the absence of shredders.

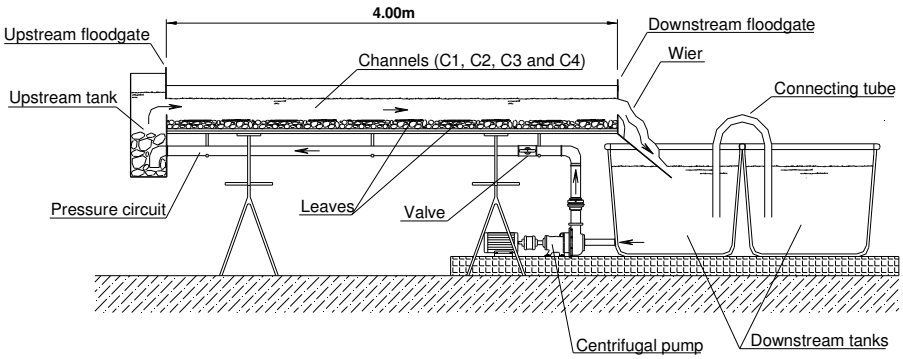
METHODS

Artificial channels, current velocity and invertebrate shredders

To assess the effects of current velocity and invertebrate activities in the fungal communities colonizing alder (*Alnus glutinosa* (L.) GAERTNER) leaves, 4 acrylic contiguous artificial channels were used, where current velocity and shredder presence (*Sericostoma* sp.) were manipulated (**Fig. 1**; for more details on the channels see FERREIRA *et al.*, 2006).

As an *inoculum* of aquatic fungi, conditioned leaves (approximately 20 g) were collected from a local stream (S. João stream, Lousã, Central Portugal, 40º05'59'' N, 8º14'02'' W) and placed in the upstream tank that delivered water to the channels (**Fig. 1**). Water (1 m^3) was also collected from the same stream. Current velocity (measured by the volumetric method) was set to match the maximum measured in zones of the stream where there was organic matter accumulation (0.2 m s^{-1}; channels C3 and C4) and to a low value (0.05 m s^{-1}; channels C1 and C2).

1a



1b

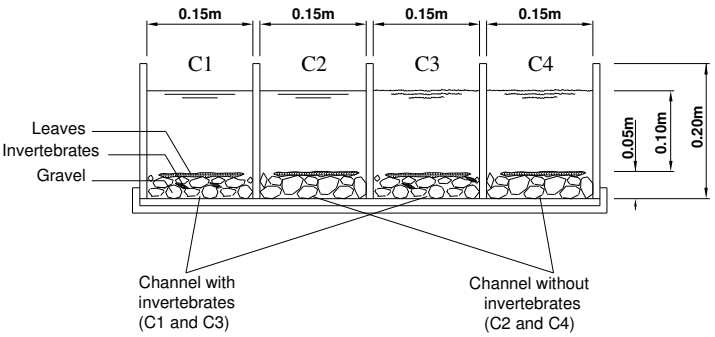


Figure 1. Scheme of the artificial channels used in the laboratory experiments in lateral (**a**) and cross-section (**b**) views. The arrows show the direction of flow in the hydraulic circuit. Channels C1 and C2 had low current velocity while channels C3 and C4 had high current velocity. Channels C1 and C3 had shredders.

In one low (C1) and one high (C3) current velocity channels, 100 individuals of the shredder *Sericostoma* sp. (Trichoptera: Sericostomatidae) were added so as to match densities observed in the field. The animals were collected from two streams (S. João stream, Lousã and Castelões stream, Caramulo) and they were in the laboratory for at least one week before being placed in the channels (for more details see FERREIRA *et al*., 2006).

Litter bags and decomposition

Leaves were collected from the same group of trees in Varandas do Ceira (Portugal, 40º10'20'' N, 8º18'10'' W) just after abscission (November 4–12, 2002). They were air dried and stored until needed. Batches of 0.74–1.00 g (mean=0.86 g) of air-dry leaves were rehydratated and placed in fine mesh (FM; 10 x 15 cm, 0.5 mm mesh size; to prevent invertebrate access to leaves) and coarse mesh (CM; 10 x 15 cm, 10 mm mesh size; to allow invertebrate access to leaves) bags. Five fine mesh and 5 coarse mesh bags were placed in each of four contiguous artificial channels. Six bags were set apart to determine initial air dry mass to ash free dry mass (AFDM) conversion factor.

After 7, 14, 21, and 28 days in water, one fine mesh and one coarse mesh bag were retrieved from each channel. To maintain the same amount of organic matter in the channels, each retrieved bag was replaced with a new one. To maintain the density of invertebrates in the channels, all the individuals that were attached to the leaves were removed and placed again in the same channel and new larvae replaced all the pupae and empty cases. Physico-chemical parameters of the water were also monitored including pH (Jenway 3310), conductivity and temperature (WTW LF 330). Water was filtered (Millipore APFF), stored in acid washed plastic bottles on ice, and analyzed for alkalinity (by titration to an end point of 4.5; APHA, 1995). A subsample was frozen for later determination of NO_3 (by ion chromatography; Dionex DX-120) and soluble reactive phosphorus (SRP; by the ascorbic acid method; APHA, 1995).

Leaves retrieved from the artificial channels were rinsed with distilled water and 5 leaf disks from each bag were cut out with a cork borer (12 mm diameter) to induce sporulation by aquatic hyphomycetes (see below). Leaf remains (including leaf disks after sporulation) were placed in an oven at 70 ºC for 72 h, weighed, ashed at 550 ºC for 4 h and reweighed to calculate remaining AFDM. The experiment was repeated three times (1st run on February, 2nd on March and 3rd on April, 2003), to generate replicates. Between each run, water, conditioned leaves and animals were changed and stones, sand and channels were clean.

Aquatic hyphomycete sporulation

Leaf disks were incubated in 100 mL Erlenmeyer flasks with 25 mL of filtered channels water (glass fiber filter, Millipore APFF), on an orbital shaker (100 rpm) for 48 h at 15 ºC to induce sporulation by aquatic hyphomycetes (BÄRLOCHER, 2005). The conidia suspensions were fixed with 2 mL of 37 % formalin for later counting and identification. When preparing

slides, 100 µL of 0.5 % Triton X-100 solution were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot of the suspension was filtered (Millipore SMWP, pore size 5 µm). Filters were stained with 0.05 % cotton blue in lactic acid, and spores were identified and counted with a compound microscope at 200x. Leaf disks were used to calculate remaining AFDM (as above). Sporulation rates were expressed as number of conidia released mg^{-1} AFDM day^{-1}.

DATA ANALYSIS

Comparisons of water parameters among the three experimental runs were made by 1-way ANOVA. When data were not normally distributed (Shapiro-Wilk's test) they were log transformed (ZAR, 1999). Percentage of AFDM remaining (arcsine square root transformed) of alder leaves after 28 days of incubation in the channels was compared among mesh types and channels by 3-way ANOVA with mesh type, current velocity and shredders as categorical variables.

Sporulation rates and species richness of aquatic hyphomycetes on alder leaves along time were compared among mesh types and channels by 4-way ANOVA with mesh type, current, shredders and time as categorical variables. The number of species colonizing leaves on day 7 was also compared among mesh types and channels by 3-way ANOVA with mesh type, current and shredders as categorical variables. Cumulative conidial production at each sampling date was calculated by summing up values of daily production at each sampling date and linearly approximated values for each day between sampling dates. Comparisons among slopes were made by 4-way ANCOVA with mesh type, current and shredders as categorical variables and time as continuous variable. Data was log transformed when necessary to achieve normality (ZAR, 1999).

Since different fungal *inocula* were used in the three runs, individual fungal species responses to current velocity and shredder activity were compared in the 3 runs individually and a species was considered to be sensitive to current velocity or shredder activity when it responded in the same way in all three runs. Differences between treatments were assessed by paired t test.

Simpson's index,

$$D = \sum_{i=1}^{S} \frac{n_i (n_i - 1)}{N (N - 1)}$$

where n_i is the number of individuals of species i, N is the total number of individuals and S the total number of species, was also calculated as a measure of heterogeneity and gives the probability of two individuals chosen at random and independently from the population belonging to the same species (WASHINGTON, 1984). Comparisons (arcsine square root transformed data) among mesh types and channels were made by 4-way ANOVA with mesh type, current, shredders and time as categorical variables. Statistical analyses were performed with STATISTICA 6 software.

RESULTS

Water parameters, shredders and mass loss

Conditions in all measured water parameters (except current velocity) were not statistically different among the three experimental runs (1-way ANOVA, $p>0.062$; **Table 1**). In spite of higher current velocities in the 1st run (1-way ANOVA, $p<0.001$), the ratio between the high and the low current velocity channels was always 5 (**Table 1**).

Table 1. Mean (SD) chemical and physical parameters of water in channels, in the 3 runs (*n*=6, except in *, where only one measurement was made).

	1st run	2nd run	3rd run
pH	7.2 (0.1)	7.0 (*)	7.1 (*)
Alkalinity (mg $CaCO_3$ L^{-1})	12.9 (1.4)	11.4 (*)	-
Conductivity (µS cm^{-1})	59.1 (4.6)	58.4 (6.1)	62.4 (6.4)
NO_3-N (µg L^{-1})	712.2 (154.8)	1130.0 (314.1)	1521.5 (412.5)
SRP (µg L^{-1})	4.2 (1.2)	6.5 (1.6)	2.3 (0.6)
Temperature (ºC)	22.4 (0.2)	22.9 (0.2)	23.2 (0.2)
Current velocity (m s^{-1})			
Channel C1	0.05 (0.0004)	0.04 (0.0007)	0.04 (0.0005)
Channel C2	0.05 (0.0004)	0.04 (0.0005)	0.04 (0.0007)
Channel C3	0.25 (0.0006)	0.17 (0.0026)	0.17 (0.0021)
Channel C4	0.25 (0.0006)	0.17 (0.0027)	0.17 (0.0016)

During the experiment some sericostomatids were lost due to pupation and, in spite of larvae replacement, by the end of each run the difference in the number of shredders between C1 and C3 was1–16 %.

Mass loss was significantly higher for alder leaves incubated in channels with shredders (C1 and C3) than in channels without shredders (C2 and C4; 3-way ANOVA, $p=0.004$) but only in coarse mesh bags (3-way ANOVA, $p=0.002$) (**Table 2** and **3**). No significant differences were found between bags exposed to different current velocities (3-way ANOVA, $p=0.186$; **Table 2** and **3**). For more details on the effect of current velocity and invertebrate

activity on decomposition of alder leaves in artificial channels and stream conditions see FERREIRA *et al*. (*in press*).

Table 2. Remaining AFDM (%) of alder leaves incubated in coarse mesh (CM) and fine mesh (FM) bags in channels 1 (C1), 2 (C2), 3 (C3) and 4 (C4) after 28 days, in the 3 runs.

	Current	Shredders	1st run	2nd run	3rd run
CM- C1	Low	Yes	32	33	41
FM- C1	Low	Yes	53	76	60
CM- C2	Low	No	59	64	64
FM- C2	Low	No	62	61	60
CM- C3	High	Yes	6	36	38
FM- C3	High	Yes	55	59	62
CM- C4	High	No	62	53	57
FM- C4	High	No	57	62	55

Table 3. Results from the 3-way ANOVA (mesh type, current velocity and shredders as categorical variables) on remaining AFDM of alder leaves incubated in the artificial channels for 28 days.

	df	SS	MS	F ratio	p
Intercept	1	404.413	404.413	1839.096	0.000
Mesh type	1	2.762	2.762	12.562	0.003
Cvel	1	0.420	0.420	1.910	0.186
Shredders	1	2.511	2.511	11.417	0.004
Mesh type*Cvel	1	0.108	0.108	0.490	0.500
Mesh type*Shredders	1	2.853	2.853	12.973	0.002
Cvel*Shredders	1	0.079	0.079	0.357	0.558
Mesh type*Cvel*Shredders	1	0.056	0.056	0.256	0.620
Error	16	3.518	0.220		

Fungal community

Twenty samples (the majority from day 7) produced less than 100 conidia mg^{-1} AFDM day^{-1} and only 10 samples produced more than 1000 conidia mg^{-1} AFDM day^{-1}. A maximum of 4025 conidia mg^{-1} AFDM day^{-1} was recorded from fine mesh bags in channel 4 (high current, no shredder) on day 7 from the 2nd run. In general, sporulation pattern was similar for most bag types. The sporulation rate increased until days 14 or 21, decreasing thereafter (**Fig. 2**). The high variability (SE bars) found for fine and coarse mesh bags in C4 (high current, no shredder) on days 7 and 14, respectively, was due to the high sporulation these bags presented on the 2nd run. Sporulation rate was higher for leaves in high than in low current channels (4-way ANOVA, $p<0.001$) but no difference was found between channels with and without shredders (4-way ANOVA, $p=0.265$) (**Table 4**).

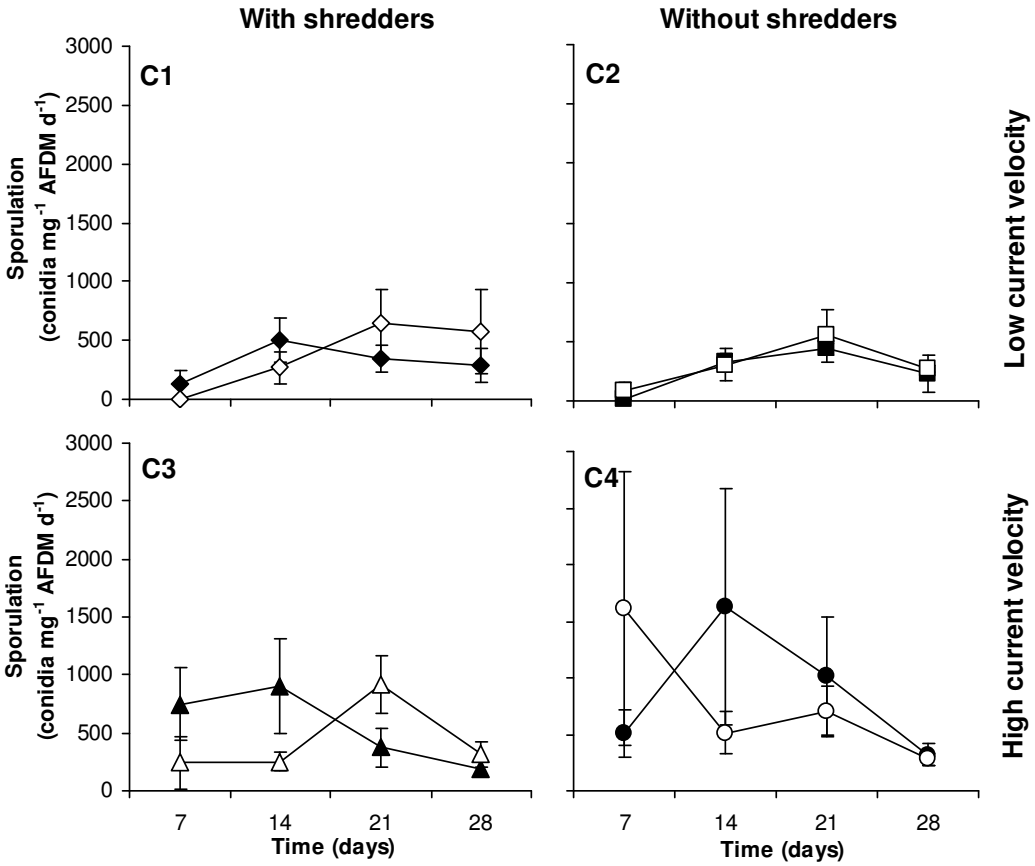


Figure 2. Sporulation rate (mean±1SE) by aquatic hyphomycetes colonizing alder leaves incubated in coarse mesh (solid symbols) and fine mesh (open symbols) bags in channels C1, C2, C3 and C4.

Table 4. Results from the 4-way ANOVA (mesh type, current velocity, shredders and time as categorical variables) on the sporulation by aquatic hyphomycetes colonizing alder leaves incubated in artificial channels.

	df	SS	MS	F ratio	p
Intercept	1	538.088	538.088	2399.110	0.000
(1)Mesh type	1	0.019	0.019	0.083	0.774
(2)Cvel	1	4.394	4.394	19.592	0.000
(3)Shredders	1	0.284	0.284	1.265	0.265
(4)Time	3	9.527	3.176	14.159	0.000
Mesh type*Cvel	1	0.120	0.120	0.537	0.466
Mesh type*Shredders	1	0.618	0.618	2.756	0.102
Cvel*Shredders	1	0.501	0.501	2.235	0.140
Mesh type*Time	3	1.129	0.376	1.678	0.181
Cvel*Time	3	5.851	1.950	8.696	0.000
Shredders*Time	3	0.325	0.108	0.482	0.696
Mesh type*Cvel*Shredders	1	0.158	0.158	0.707	0.404
Mesh type*Cvel*Time	3	0.108	0.036	0.161	0.922
Mesh type*Shredders*Time	3	3.149	1.050	4.680	0.005
Cvel*Shredders*Time	3	0.149	0.050	0.221	0.881
1*2*3*4	3	0.010	0.003	0.015	0.997
Error	64	14.354	0.224		

Cumulative conidial production was also significantly higher in leaves exposed in high than in low current channels (4-way ANCOVA, $p<0.001$; **Fig. 3** and **Table 5**). In channel C3 (high current, with shredders) there was higher conidial production in coarse than in fine mesh bags (4-way ANCOVA, $p=0.023$), however, considering the 4 channels, there was no effect of shredders or mesh type in the conidial production (4-way ANCOVA, $p>0.337$) (**Table 5**). The higher values found for leaves in C4 (high current, no shredders) were, to a great extent, due to the high sporulation registered in the 2nd run.

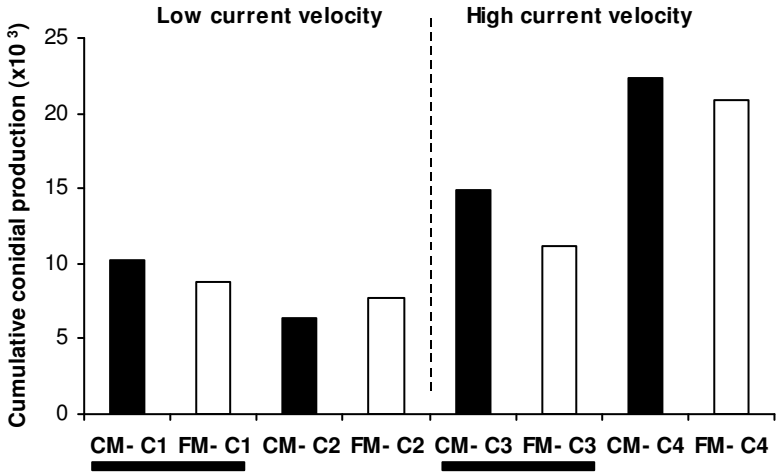


Figure 3. Cumulative conidia production by aquatic hyphomycetes colonizing alder leaves incubated in coarse mesh (CM) and fine mesh (FM) bags in channels C1, C2, C3 and C4 after 28 days. Horizontal solid bars indicate shredder presence.

Table 5. Results from the 4-way ANCOVA (mesh type, current velocity and shredders as categorical variables and time as continuous variable) on the cumulative conidial production by aquatic hyphomycetes colonizing alder leaves incubated in artificial channels.

	df	F ratio	p
Intercept	1	454.060	0.000
Time	1	50.468	0.000
Mesh type	1	0.725	0.394
Cvel	1	25.511	0.000
Shredders	1	0.924	0.337
Mesh type*Cvel	1	0.000	0.987
Mesh type*Shredders	1	5.179	0.023
Cvel*Shredders	1	1.520	0.218
Mesh type*Cvel*Shredders	1	0.376	0.540

A minimum of 2 and a maximum of 11 species of aquatic hyphomicetes were identified from samples retrieved from the channels. The dynamics of species colonization was similar to sporulation, with a peak in species richness by day 14–21 (**Fig. 4**). No significant differences were found between mesh types (4-way ANOVA, $p=0.726$; **Table 6**). Leaves

incubated in high current channels had higher species richness than leaves incubated in low current channels (4-way ANOVA, p=0.011) but again no difference was found between channels with and without shredders (4-way ANOVA, p=0.985) (**Table 6**). No significant differences were found in the number of fungal species colonizing leaves on day 7 that could be attributed to either mesh type, current or shredders (3-way ANOVA, p>0.208).

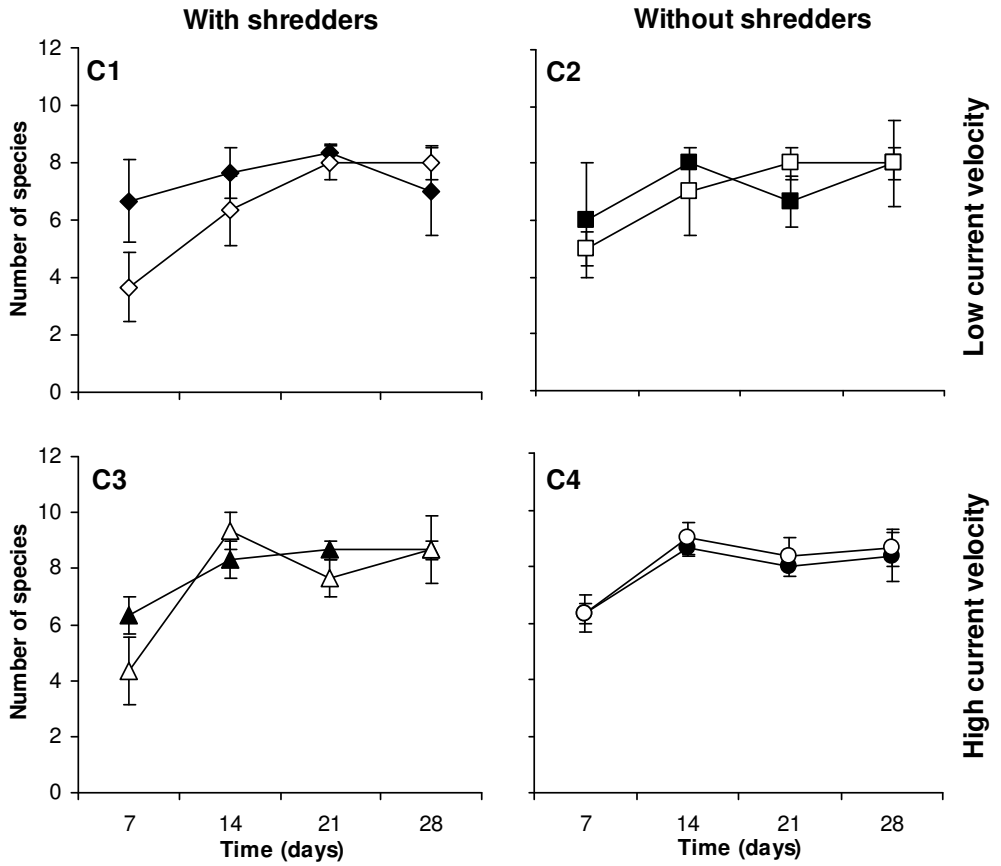


Figure 4. Number of species (mean±1SE) of aquatic hyphomycetes colonizing alder leaves incubated in coarse mesh (solid symbols) and fine mesh (open symbols) bags in channels C1, C2, C3 and C4.

There was no consistent difference in species identity among mesh types and channels for the 3 runs (data not shown). The same was observed for species abundance; except for *Tetrachaetum elegans*, *Tricladium chaetocladium* and *Triscelophorus monosporus*, which were more abundant in high current channels, being the reverse true for *Lunulospora curvula* (paired t test, p<0.003; **Table 7**). In the 1st run, the dominant species were *Triscelophorus acuminatus* (maximum of 88 % of spores produced per sample), *L. curvula* (maximum of 82 %) and *T. elegans* (maximum of 58 %); in the 2nd run, the dominant species were *T. chaetocladium* (maximum of 71 %), *T. elegans* (maximum of 51 %) and *L. curvula* (maximum

of 62 %); and in the 3rd run, the dominant species were *L. curvula* (maximum of 96 %) and *Anguillospora filiformis* (maximum of 52 %).

Table 6. Results from the 4-way ANOVA (mesh type, current velocity, shredders and time as categorical variables) on the number of species of aquatic hyphomycetes colonizing alder leaves incubated in artificial channels.

	df	SS	MS	F ratio	p
Intercept	1	78.563	78.563	5029.221	0.000
(1)Mesh type	1	0.002	0.002	0.124	0.726
(2)Cvel	1	0.106	0.106	6.789	0.011
(3)Shredders	1	0.000	0.000	0.000	0.985
(4)Time	3	0.623	0.208	13.297	0.000
Mesh type*Cvel	1	0.001	0.001	0.067	0.796
Mesh type*Shredders	1	0.048	0.048	3.045	0.086
Cvel*Shredders	1	0.010	0.010	0.609	0.438
Mesh type*Time	3	0.014	0.005	0.302	0.824
Cvel*Time	3	0.030	0.010	0.632	0.597
Shredders*Time	3	0.005	0.002	0.108	0.955
Mesh type*Cvel*Shredders	1	0.007	0.007	0.450	0.505
Mesh type*Cvel*Time	3	0.029	0.010	0.626	0.601
Mesh type*Shredders*Time	3	0.103	0.034	2.196	0.097
Cvel*Shredders*Time	3	0.042	0.014	0.897	0.448
1*2*3*4	3	0.027	0.009	0.571	0.636
Error	64	1.000	0.016		

Table 7. Mean relative abundance (%; means over all sampling dates) of aquatic hyphomycete conidia released from alder leaves in low and high current velocity channels, in the 3 runs. +, mean relative abundance <0.1%

	1st run		2nd run		3rd run	
Species	**Low**	**High**	**Low**	**High**	**Low**	**High**
Alatospora acuminata INGOLD	0.7	1.8	0.5	0.5	0.1	0.3
Anguillospora filiformis GREATHEAD	2.0	3.6	12.3	7.3	24.1	27.0
Anguillospora longissima (SACC. ET SYD.) INGOLD			+			
Articulospora tetracladia INGOLD	20.1	1.9	0.2	0.6		0.1
Clavariopsis aquatica DE WILD.		+	0.4	0.9	0.1	0.3
Heliscus ludgunensis SACC. ET THÉRRY			+	0.1	+	
Lemonniera aquatica DE WILD.	0.2					
Lunulospora curvula INGOLD*	20.4	16.7	22.7	2.8	48.8	13.2
Tetrachaetum elegans INGOLD*	16.3	19.8	13.1	19.7	4.3	9.9
Tricladium chaetocladium INGOLD*	1.4	3.4	24.6	41.2	6.5	16.6
Triscelophorus acuminatus NAWAWI	29.7	37.5	8.7	6.3	4.6	2.9
Triscelophorus monosporus INGOLD*	2.2	9.0	5.4	6.1	4.3	12.1
Unidentified tetraradiate$	0.1	0.3	1.8	7.4	3.3	11.8
Small sigmoid (<60 µm)	7.0	6.0	4.1	7.0	4.0	5.8

*species that present a consistent response to current velocity

$conidia with broken arms, probably *T. elegans* and *T. chaetocladium*

Simpson's index was in general low (D<0.55; **Fig. 5**), which indicates that the fungal community on leaves was not clearly dominated by a single species. However, there was a current velocity effect with the fungal community in alder leaves incubated in high current rather than low current channels having lower values (4-way ANOVA, p=0.001; **Table 8**).

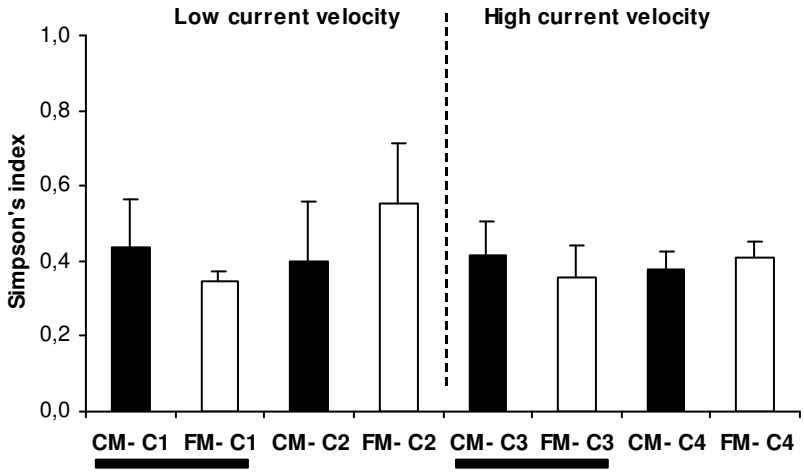


Figure 5. Simpson's index, D (mean±1SE) of the fungal communities colonizing alder leaves incubated in coarse mesh (CM) and fine mesh (FM) bags in channels C1, C2, C3 and C4 after 28 days. Horizontal solid bars indicate shredder presence.

Table 8. Results from the 4-way ANOVA (mesh type, current velocity, shredders and time as categorical variables) on the Simpson's index of the fungal communities colonizing alder leaves incubated in artificial channels.

	df	SS	MS	F ratio	p
Intercept	1	27.195	27.195	15.970	0.000
(1)Mesh type	1	0.076	0.076	0.045	0.833
(2)Cvel	1	22.692	22.692	13.326	0.001
(3)Shredders	1	2.336	2.336	1.372	0.246
(4)Time	3	1.866	0.622	0.365	0.778
Mesh type*Cvel	1	0.010	0.010	0.006	0.940
Mesh type*Shredders	1	0.138	0.138	0.081	0.777
Cvel*Shredders	1	2.407	2.407	1.414	0.239
Mesh type*Time	3	0.848	0.283	0.166	0.919
Cvel*Time	3	1.292	0.431	0.253	0.859
Shredders*Time	3	3.333	1.111	0.652	0.584
Mesh type*Cvel*Shredders	1	0.037	0.037	0.022	0.883
Mesh type*Cvel*Time	3	0.258	0.086	0.051	0.985
Mesh type*Shredders*Time	3	0.207	0.069	0.040	0.989
Cvel*Shredders*Time	3	1.867	0.622	0.365	0.778
1*2*3*4	3	0.131	0.044	0.026	0.994
Error	63	107.278	1.703		

DISCUSSION

The absence of significant differences in fungal sporulation rates, cumulative conidial production, fungal species richness and Simpson's index (heterogeneity of fungal community) that could be attributed to mesh size demonstrated that there was probably no alteration of the physical environment surrounding leaves inside fine mesh bags (0.5 mm mesh) when compared with coarse mesh bags (10 mm mesh). This was supported by the absence of significant differences in mass loss of alder leaves between coarse mesh and fine mesh bags in channels without shredders. This was opposite to what was observed by HOWE & SUBERKROPP (1994), who reported higher fungal sporulation in coarse mesh than in fine mesh bags as a result of clogging of fine mesh bags by fine particles. The difference in the effect of mesh size on the fungal community between this study and the one by HOWE & SUBERKROPP (1994) may have been due to the smaller fine mesh (0.36 mm) used by HOWE and SUBERKROPP and to the fact that their study was performed in a stream whereas this study was perform in artificial channels where the suspended sediments were absent. BÄRLOCHER found lower fungal species richness (BÄRLOCHER, 1980) and lower fungal sporulation rates (BÄRLOCHER, 1982) in coarse mesh bags but this effect of mesh size was confounded with shredder presence.

In this study there was also no effect of shredder activity on fungal parameters, and so, our results agree with those of HOWE & SUBERKROPP (1994). Although invertebrates caused a significant higher mass loss in coarse mesh bags in channels with shredders than in channels without shredders, this reduction in available substrate was not translated in a reduction of the number of species colonizing it or in a reduction of the fungal sporulation and diversity. It is therefore plausible that, at least in the case of *Sericostoma* sp., feeding does not selectively favor some species, as there was no consistent response of fungal species to shredder presence. Nevertheless, other shredders have been reported to discriminate between leaves colonized by different fungal species, when this colonization is discrete (ARSUFFI & SUBERKROPP, 1989). However, in our study each leaf was colonized by several fungal species and it is plausible that mycelia from different species of fungi may grow so intrinsically associated that discrimination would be impossible for a large size shredder. Hence, our results disagree with those of BÄRLOCHER (1980, 1982) who found a negative effect of shredder feeding on the fungal community structure as they acted either as predators on the early fungal colonizers or as competitors of the later fungal colonizers. In our channels system the number of fungal species that could colonize leaves was low and generally leaves became

fully colonized after 14 (67 % of samples) or 21 (88 % of samples) days and so shredders were probably not competitors of fungi. ROSSI (1985), on the other hand, found a positive effect of shredders on the fungal community colonizing leaf detritus what was attributed to the facilitation of fungal colonization by shredders damaging leaf epidermis. In our case this facilitation effect was not observed.

Current velocity had, however, a significant effect in the fungal parameters determined, but not in the mass loss of alder leaves. Leaves exposed in high current velocity channels had higher sporulation rates, cumulative conidial production and number of fungal species and lower Simpson's index (lower heterogeneity) than leaves exposed in low current velocity channels. Since the channels system was a closed circuit and channels were not independent, the number of conidia and species that could potentially colonize leaves was the same in all four channels. Therefore, the higher sporulation rate, cumulative conidial production and number of species in high current velocity channels could only be explained by stimulation of the sporulation itself, and not of the colonization rate. We therefore conclude that turbulence is a major factor affecting the reproductive activity of aquatic hyphomycetes as previously suggested (reviewed by BÄRLOCHER, 1992). High current velocity (i.e. high turbulence) can stimulate sporulation by (a) reducing the time required for spore development, (b) increasing the number of conidiophores per unit area (BÄRLOCHER, 1992), (c) stimulating conidia detachment or (d) facilitating nutrient acquisition. However, as sporulation from all samples was induced in the same conditions (orbital shaker at 100 rpm), it is most plausible that high current velocity in the channels had stimulated sporulation by increasing the number of conidiophores per unit area. Nutrient acquisition by fungi was probably not different between current velocity channels as the range of current velocities tested was low.

In summary, there was no evidence that mesh type or invertebrate feeding on decaying leaves affected aquatic hyphomycetes community structure. Nevertheless, in the real stream conditions it is likely to be suspended sediments that could clog fine mesh bags. On the other hand, there was higher aquatic hyphomycetes activity in channels with high current velocity when compared to channels with low current velocity, particularly due to the stimulation of certain species. Thus, in studies where the fungal community structure on decomposing litter in streams is to be addressed careful should be taken regarding the current conditions where the litter is going to be emerged.

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CHAPTER VI

FUNGAL ACTIVITY ASSOCIATED WITH DECOMPOSING WOOD IS AFFECTED BY NITROGEN CONCENTRATION IN WATER

FERREIRA V. & GRAÇA M.A.S. Fungal activity associated with decomposing wood is affected by nitrogen concentration in water. *Internat Rev Hydrobiol* (submitted)

Photos:

Balsa (*Ochroma pyramidale* (CAV. EX LAM.) URB.) squares in fine mesh bags

Balsa squares incubating in Margaraça stream (Margaraça forest, Açor mountain, Portugal)

Balsa squares in microcosms with two N concentrations

Balsa squares microcosms on an orbital shaker

FUNGAL ACTIVITY ASSOCIATED WITH DECOMPOSING WOOD IS AFFECTED BY NITROGEN CONCENTRATION IN WATER

ABSTRACT

Here we examined the effect of two contrasting nitrogen concentrations in water (0.16 and 0.82 mg N L^{-1}) in the mass loss of pre-conditioned balsa wood in a stream for 52 days, and associated fungal activity, incubated in laboratory microcosms. Contrary to expect, given the poor nutrient quality of balsa wood, the increase in dissolved nitrogen concentration did not result in increased mass loss in the laboratory for 24 days (16 % in both nitrogen concentrations). In the same way, microbial oxygen consumption was similar between both nitrogen concentrations (approx. 0.17 mg O_2 g^{-1} AFDM h^{-1}). Conidial production was stimulated in high than in low nitrogen microcosms (32 902 *vs*. 20 980 conidia $microcosm^{-1}$ by d24), although the number of species of aquatic hyphomycetes was similar between both treatments. No species was sensitive to the nitrogen concentration in microcosms. The results confirm the notion that reproductive activity of aquatic hyphomycetes is the most sensitive microbial parameter to changes in the environment.

Key-words: balsa, decomposition, fungi, microcosms, nitrogen, sporulation

INTRODUCTION

Aquatic hyphomycetes are important players in litter decomposition in freshwaters (GESSNER & CHAUVET, 1994; BALDY *et al*., 1995; HIEBER & GESSNER, 2002; PASCOAL *et al*., 2005) and their activity has been related to the nutrient concentration in water, particularly sporulation (SUBERKROPP, 1998; FERREIRA *et al*., 2006b) and oxygen consumption (STELZER *et al*., 2003; GULIS *et al*., 2004). High fungal activity can be translated into high decomposition rates as high amount of initial mass is converted into fungal mycelium and conidia (NIYOGI *et al*., 2003; GULIS & SUBERKROPP, 2003; PASCOAL *et al*., 2005; FERREIRA *et al*., 2006b). This stimulation is higher in low quality (low nutrients, high lignin) substrates (STELZER *et al*., 2003; GULIS *et al*. 2004; but see FERREIRA *et al*., 2006b).

Wood, being a highly recalcitrant substrate, is usually colonized by fewer species of aquatic hyphomycetes and has lower microbial activity when compared with leaves (SIMON & BENFIELD, 2003; STELZER *et al*., 2003; FERREIRA *et al*., 2006b). Nevertheless, when the concentration of dissolved nutrients is high, wood can be an important resource for aquatic food webs when leaves are not available due to their faster decomposition rates or because of seasonal constrains.

The objective of this study was to assess the effect of two contrasting nitrogen levels in the decomposition rates of balsa wood and in the activity (sporulation and oxygen consumption rates) of the associated aquatic hyphomycete communities, in laboratory microcosms. In a broad context, the experiment will elucidate relationships between nutrient levels in the environment and the functional process of litter decomposition

METHODS

Conditioning of balsa wood in stream and incubation in microcosms

Balsa (*Ochroma pyramidale* (CAV. EX LAM.) URB.) veneers (100 x 10 x 0.1 cm) were bought from a local supplier and cut into 1.1 cm^2 squares. Each sample was composed by 10 squares (0.1688–0.3547 g) that were placed in fine mesh bags (3 x 3 cm; 0.5 mm mesh) and incubated in Margaraça stream from April 14 to June 6, 2006. Margaraça stream is a 1st order, circumneutral, SRP rich, N limited stream running through a native deciduous forest (Margaraça Forest, Central Portugal, 40º13' N, 7º56' W). For more information about the stream see ABELHO & GRAÇA (1998) and FERREIRA *et al.* (2006b).

After the 52 days of incubation the bags containing the veneers (116 in total) were retrieved from the stream, placed in zip lock bags and transported in an ice chest to the

laboratory where they were gently rinsed with distilled water. Fifty height samples were autoclaved at 120 ºC for 15 min. Ten of these samples were oven dried at 105 ºC for 24 h, weighed, ashed at 550 ºC for 6 h and reweighed to calculate initial air-dry mass to conditioned ash free dry mass (AFDM) conversion factor. The other 48 autoclaved samples were split into 2 groups and placed in individual 100 mL Erlenmeyer flasks with 25 mL of stream water (normal N treatment; 0.16 mg N L^{-1}) or 25 mL of N adjusted stream water (high N treatment; 0.82 mg N L^{-1}; see below). These samples served as controls for fungal activity in the normal and high N concentration microcosms. The remaining non-autoclaved 58 samples were distributed as above and were used to determine the effect of two contrasting N concentrations in fungal activity. Microcosms were incubated in shakers (100 rpm) at 15 ºC for 4–24 days. The stream water used in microcosms was collect at the same time as balsa samples, transported to the laboratory, filtered (glass fiber filter, Millipore APFF) and half was used in the normal N treatment and half had its N concentration adjusted with $NaNO_3$ so that it would be approx 5x higher than normal and used in the high N treatment. Chemical composition of water was determined from filtered samples by ion chromatography (Dionex DX-120; SRP was determined by the ascorbic acid method; APHA, 1995; **Table 1**).

Table 1. Water chemistry in microcosms (mean±1SD; *n*=3).

	Normal	High
NO_3-N (mg L^{-1})	0.16 ± 0.03	0.82 ± 0.08
NH_4-N (mg L^{-1})	0.05 ± 0.00	0.03 ± 0.03
SRP (mg L^{-1})	0.07 ± 0.01	0.07 ± 0.03
Na (mg L^{-1})	7.74 ± 0.19	9.21 ± 0.56
Mg (mg L^{-1})	3.64 ± 0.03	3.78 ± 0.23
Ca (mg L^{-1})	3.77 ± 1.13	5.69 ± 4.75
K (mg L^{-1})	0.51 ± 0.25	0.45 ± 0.15
Cl (mg L^{-1})	9.12 ± 7.42	5.27 ± 0.82
SO_4 (mg L^{-1})	6.25 ± 2.38	4.85 ± 0.57

The solutions in the microcosms were replaced every 4 days and 4 replicate microcosms of each of the 4 treatments (control+normal N, control+high N, conditioned+normal N and conditioned+high N) were scarified for measurements (see below). Fungal activity was determined only from conditioned+normal N and conditioned+high N treatments while mass loss was determined from all 4 treatments.

Aquatic hyphomycete sporulation and microbial respiration

The conidia suspensions of conditioned+normal N and conditioned+high N treatments were decanted in 50 mL centrifuge tubes, and conidia fixed with 2 mL of 37 % formalin to be later counted and identified. When preparing slides for conidia identification, 100 µL of Triton X-100 solution (0.5 %) were added to the suspension to ensure a uniform distribution of conidia, stirred and an aliquot of the suspension was filtered (Millipore SMWP, 5 µm pore size). Filters were stained with cotton blue in lactic acid (0.05 %), and spores were identified and counted with a compound microscope at 200x. Values were expressed as number of conidia microcosm^{-1}.

The 10 balsa squares of conditioned+normal N and conditioned+high N treatments sacrificed every 4 days were used to determine microbial oxygen consumption rates (ABELHO & GRAÇA, 2000). The flow-through system set at 15 ºC consisted of a peristaltic pump with adjustable flow provided with Watson-Marlow orange/green tubes. One end of the tubes was connected to the respiration chambers (8 ml glass syringes) and the other end entered a reservoir containing normal or high N concentration stream water 100 % oxygenated. Measurements of oxygen concentrations in water were made only after the chambers' volume was totally replaced. The water flowing through the chambers was collected with a 1 mL syringe and injected into a 0.1 mL micro-chamber adapted to an oxygen electrode (Strathkelvin Inst. 781) and readings made after 30 seconds. After 3 measurements the flow was determined with 5 mL calibrated glass vials for 20 minutes. Oxygen consumptions were expressed as mg O_2 g^{-1} AFDM h^{-1}.

Decomposition

After the respirometry trial balsa squares of conditioned+normal N and conditioned+high N treatments were oven dried at 105 ºC for 24 h, weighed, ashed at 550 ºC for 6 h and reweighed to calculate AFDM remaining. Balsa squares of control+normal N and control+high N treatments were also used to determine AFDM remaining.

DATA ANALYSIS

Differences in remaining AFDM by day 24 between treatments were assessed by 2-way ANOVA with N concentration (normal and high) and balsa type (control and conditioned) as categorical variables.

Microbial oxygen consumption, aquatic hyphomycetes sporulation rates and species richness and % contribution by selected fungal species to the total conidia production were compared between normal and high N microcosms by 2-way ANOVA (N concentration and time as categorical variables). Cumulative conidial production at each sampling date was calculated by summing up values of daily production at each sampling. Comparison of cumulative conidial production between treatments was done by ANCOVA (N concentration as categorical variables and time as continuous variable). Data was transformed when necessary to achieve normality (ZAR, 1999) and analyses were performed with STATISTICA 6 software.

RESULTS

Decomposition

After 52 days in the stream, balsa squares lost *ca*. 40 % of their initial mass. The remaining mass was considered the initial mass for the experiment in microcosms. After 24 days in microcosms conditioned balsa squares lost 16 % of their initial mass in both N treatments while control balsa squares lost from 2 % (normal N) to 12 % (high N) of their initial mass (**Fig. 1**). By day 24 no significant difference was found between veneers incubated in the 2 N concentrations (2-way ANOVA, p=0.807) or balsa type (2-way ANOVA, p=0.218).

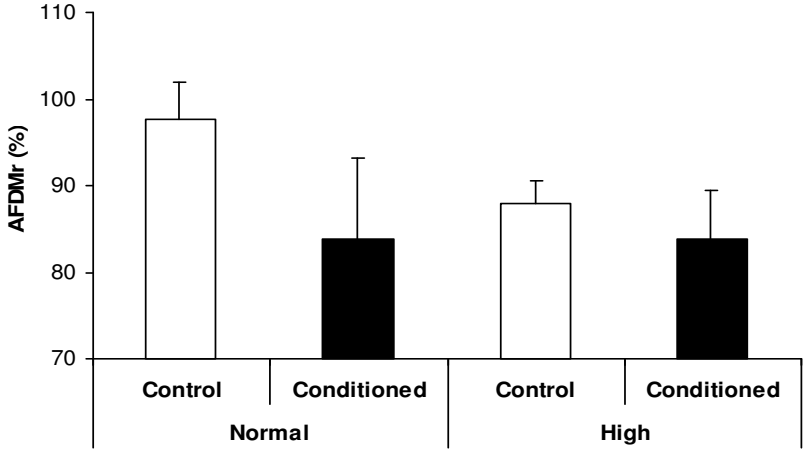

Figure 1. Remaining mass after 24d in microcosms (average±1SE) of control and conditioned balsa squares incubated in normal and high N microcosms.

Microbial oxygen consumption

Oxygen consumption by microbes associated with balsa squares peaked by day 12 in the laboratory in high N microcosms (0.26 mg O_2 g^{-1} AFDM h^{-1}) while it decreased over time

in normal N microcosms (0.27 to 0.18 mg O_2 g^{-1} AFDM h^{-1} from d4 to d24) (**Fig. 2**). However, no significant differences in respiration rates between N concentrations were found (2-way ANOVA, p=0.549).

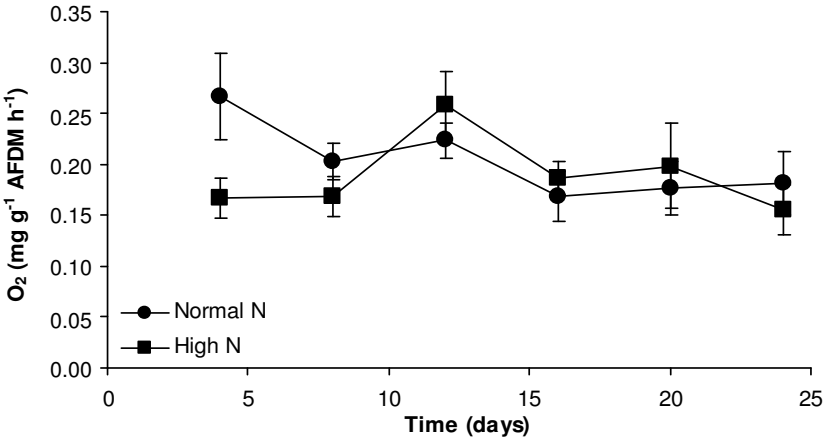

Figure 2. Microbial oxygen consumption (average±1SE) in conditioned balsa squares incubated in normal and high N microcosms.

Aquatic hyphomycete sporulation

Conidia production increased through time and was always higher in high N microcosms (32 902 *vs*. 20 380 conidia microcosm^{-1} by d24; 2-way ANOVA, p=0.001; **Fig. 3a**). Cumulative conidia production was up to 2 times higher in high than in normal N microcosms (108 591 *vs*. 59 260 conidia by d24; ANCOVA, p=0.008; **Fig. 3b**).

There were 6 species of aquatic hyphomycetes sporulating in balsa squares in microcosms but *Anguillospora crassa* dominated the fungal communities in all sampling dates in both N concentration microcosms (75–96 % contribution to the total conidial production; **Table 2**). The number of species sporulating in balsa squares was similar between normal and high N microcosms (2-way ANOVA, p=0.659) and it decreased through time (**Fig. 3c**). There were no sensitive species to the N concentration in microcosms (2-way ANOVA, p=0.103–0.696).

Table 2. Mean relative abundances (%, over all sampling dates) of aquatic hyphomycete conidia in balsa squares incubated in normal and high N microcosms.

	Normal N						High N					
Days in water	4	8	12	16	20	24	4	8	12	16	20	24
Alatospora acuminata	8.2	10.6	13.5		0.9	0.7	1.8	7.1	1.0	2.0	5.1	0.6
Anguillospora crassa	75.6	74.8	74.2	96.1	96.3	95.0	85.4	74.8	94.1	94.8	92.0	94.2
Clavariopsis aquatica	3.1	8.7	8.3	3.4	2.4	2.7	5.7	14.1	3.5	0.8	2.1	5.1
Tetrachaetum elegans	0.2		0.5			0.7						
Tricladium chaetocladium	1.9	1.3	0.6		0.1	1.0	1.2	1.1	0.2	0.9	0.6	
Tricladium splendens	11.0	4.6	2.9	0.6	0.3		5.9	2.9	1.1	1.5	0.3	0.2

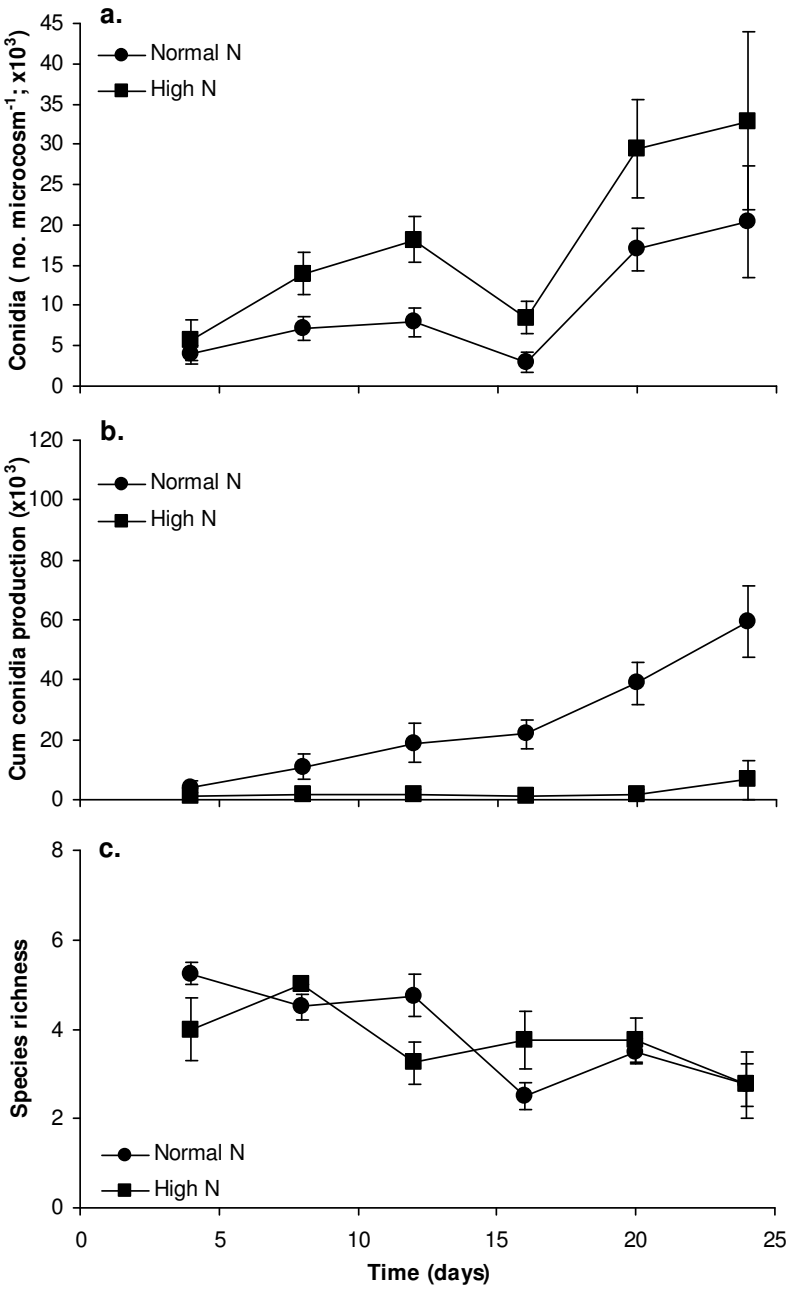

Figure 3. Conidia (average±1SE; **a**), cumulative conidia production (average±1SE; **b**) and species richness (average±1SE; **c**) of aquatic hyphomycetes associated with conditioned balsa squares incubated in normal and high N microcosms.

DISCUSSION

Given the low nutrient quality of balsa wood (FERREIRA *et al*., 2006b) it was expected that an increase in dissolved nitrogen would lead to an increase in mass loss and associated microbial

activity (DÍEZ *et al*., 2002; GULIS *et al*., 2004). This was most expected as dissolved phosphorus was present in high quantity and previous field experiments showed that nitrogen was the limiting nutrient for fungal activity associated with this woody substrate (FERREIRA *et al*., 2006b). However, decomposition of balsa was not sensitive to nitrogen concentration in microcosms. During the 52 days conditioning period in the stream balsa squares lost *ca*. 40 % of their initial mass, which was higher than reported in a previous experiment (*ca*. 25 %; FERREIRA *et al*., 2006b; **Fig. 4**). This difference could be attributed to the season in which decomposition trials were carried out; in this study balsa conditioning was carried out in late spring while FERREIRA *et al*. (2006b) performed their studies in autumn/winter when the water temperature is lower. Stimulation of litter decomposition and microbial activity by increased water temperature was reported before (CHERGUI & PATTEE, 1990; CHAUVET & SUBERKROPP, 1998; FERREIRA *et al*. 2006a). Anyway, as decomposition of balsa wood was accelerated regarding previous experiment, when balsa squares were transferred to microcosms they were probably entering the phase of lower mass loss rate (**Fig. 4**). This could in part explain the absence of a stimulation of mass loss in high nitrogen microcosms. The lack of significant differences in mass loss between control and conditioned balsa squares, although unexpected, could be explained by higher desegregation of fibers from wood after being autoclaved than from non-autoclaved squares.

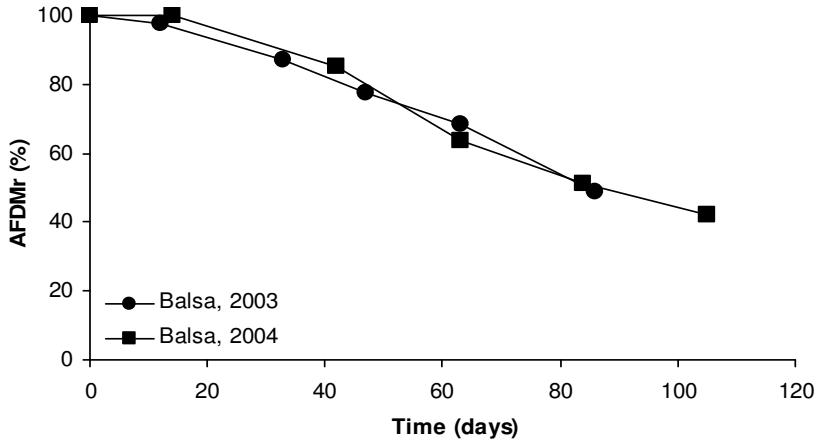

Figure 4. Remaining mass (average±1SE) of balsa veneers incubated in a reference site (NO_3-N=104±40 µg L^{-1} and SRP=104±34 µg L^{-1}, average±SD) at Margaraça stream in autumn/winter 2003 and 2004 (site R; FERREIRA *et al*., 2006b).

Similarly, no significant differences were found in microbial oxygen consumption between microcosms with contrasting nitrogen concentrations. This was also unexpected as several studies reported a stimulation of overall microbial activity in the presence of increased

dissolved nutrients (GULIS & SUBERKROPP, 2003; NIYOGI *et al*., 2003; STELZER *et al*., 2003; GULIS *et al*., 2004).

Balsa squares were colonized by only 6 species of aquatic hyphomycetes against the 11 that were recorded colonizing this substrate in the same stream site (site R; FERREIRA *et al*., 2006b). The difference was probably a result of balsa wood being incubated in spring in this study while FERREIRA *et al*. (2006b) incubated it in two following autumns with several sampling dates. The number of species sporulating in balsa squares was similar between microcosms with contrasting nitrogen concentration what is not surprising since in the stream these species were present in a wide range of nitrogen concentrations (approx 100–1000 μg L^{-1}; FERREIRA *et al*., 2006b). On the other hand, the percentage contribution of each species to the total conidia production was similar in both treatments for all species, which for *Anguillosora crassa* and *Clavariopsis aquatica* was surprising given previous field results. *C. aquatica* dominated fungal communities when high nitrogen concentrations were available (983±139 μg L^{-1}) while *A. crassa* dominated in the presence of low nitrogen concentrations (82±7 μg L^{-1}) (FERREIRA *et al*., 2006b). The lack of *C. aquatica* to dominate at high nitrogen microcosms could be explained by its preference by cooler water (BÄRLOCHER, 1992). Between days 8 and 12 of the microcosms experiment the air conditioning system failed to keep the room at 15 ºC and the air temperature raised up to 21 ºC for 2 days. In this period the percentage contribution of *C. aquatica* decreased from 14 to 3.5 % in high nitrogen microcosms and remained low thereafter.

The relevant finding in this experiments was that, among all the measured parameters, only conidia production differed among treatments: conidia production by aquatic hyphomycetes was higher in high nitrogen microcosms, as reported in other studies using wood (FERREIRA *et al*., 2006b) and leaves (SUBERKROPP, 1998; GULIS & SUBERKROPP, 2003) as substrates. The data suggests that changes in the environment are likely to cause biological–physiological modifications at individual level, before ecosystem functional parameters be altered. However, more spores in the water column may increase the rate of colonization of substrates and cause an accelerated initial decomposition.

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GENERAL CONCLUSION

The experiments reported in this thesis assessed the effect of several environmental factors on the decomposition of submerged litter and associated microbes and invertebrates. The underlying goal of most experiments (Chapters I–IV) was to assess the potential for decomposition rates of submerged litter to be used as an assessment tool to evaluate the functional integrity of streams (GESSNER & CHAUVET, 2002). If decomposition rates are to be used in this way one must demonstrate that 1) they are sensitive, and respond in a predictable way, to anthropogenic impacts, and 2) they are insensitive to natural site variability. Two factors that can change between sites, and potentially affect litter decomposition, are current velocity and invertebrate density (Chapter I). Decomposition of the soft alder leaves was unaffected by low to moderate current velocities in either artificial channels or a 4th order stream. However, suspended sediments might increase the abrasive effect of current velocity. Careful should be taken when allocating litter bags in the streams, particularly in autumn when runoff due to rains increases the amount of suspended sediments in water. This is also important since shredding invertebrates are major players in litter decomposition (CUFFNEY *et al.*, 1990; STEWART, 1990; RADER *et al.*, 1994; HIEBER & GESSNER, 2002) and are usually more abundant in shallow riffles (GRAÇA *et al.*, 2004). Although litter decomposition can be sensitive to inter-habitat variability this can be overcome by allocating bags in similar conditions.

Two widespread anthropogenic disturbances to low order streams, nutrient enrichment of water and forest change, were used to test the efficiency of decomposition rates in assessing stream functional integrity. Anthropogenic nutrient enrichment of streams and rivers, by either domestic sewage or agriculture runoff, is the most widespread cause of aquatic pollution (SWEETING, 1996). In our experiments, decomposition rates of oak leaves and balsa veneers (but not of alder leaves) were stimulated by higher nutrient (N and/or SRP) concentration in water (Chapters II and III). The stimulation of mass loss in the poor quality litter with increasing availability of dissolved nutrients resulted from a stimulation of the microbial activity (Chapter III), which was expected (GULIS & SUBERKROPP, 2003) as fungi can obtain nutrient from both the substrate and surrounding water (SUBERKROPP, 1998). Of the three metrics proposed by GESSNER & CHAUVET (2002) to assess stream functional integrity, a) absolute k values, b) k_{CM}: k_{FM} and c) $k_{impacted}$: $k_{reference}$, the $k_{impacted}$: $k_{reference}$ was the most

sensitive to water quality, in particular when applied to the poor quality oak leaves incubated in coarse mesh bags (Chapter II). Metrics and indices, including the biotic index IBMWP, based on the benthic macroinvertebrate community, traditionally used in biological assessments were not very useful evaluating streams structural integrity, with the exception of some metrics related with Plecoptera and shredders (Chapter II). However, the overall benthic invertebrate community was able to discriminate reference from nutrient enriched streams (Chapter II).

In Central Portugal and North Spain many streams are also impaired by the substitution of the native deciduous riparian vegetation by the evergreen *Eucalyptus globulus* (reviewed by GRAÇA *et al*., 2002; GRAÇA & CANHOTO, 2006). The effect of eucalyptus plantations in streams has been extensively studied in both countries, but some results are contrasting between the two regions (reviewed by GRAÇA *et al*., 2002). For the first time (Chapter IV), the same protocol was applied to study the effect of the substitution of native deciduous forest by eucalyptus plantations in Portuguese and Spanish streams. Decomposition of alder and oak leaves was similar between eucalyptus and deciduous streams, although in Portugal the fungal community structure associated with submerged substrates discriminated stream types much better than in Spain. This might indicated a certain degree of functional redundancy among decomposers, as changes in community structure were not translated into changes in decomposition rates. Also, the effect of changes in riparian vegetation was higher in Portugal than in Spain probably as a result of differences in climate, which is wetter in Spain allowing for the development of an understory of deciduous trees in eucalyptus plantations that increases the diversity of the litter input to streams. To mitigate the effect of changes in timing, quality and quantity of litter inputs to streams running through eucalyptus plantations (ABELHO & GRAÇA, 1996; POZO *et al*., 1997a, b; MOLINERO & POZO, 2003, 2004) in the stream biota deciduous trees should be present in the riparian zone.

Aquatic fungi are the key players in litter decomposition as they convert litter carbon into biomass and conidia (SUBREKROPP & CHAUVET, 1995; GULIS & SUBREKROPP, 2003b) and enhance litter quality for shredders (ARSUFFI & SUBERKROPP, 1985, 1986; GRAÇA *et al*., 2001). However, the effect of shredders on aquatic hyphomycete community structure and activity is not clear (BÄRLOCHER, 1980, 1982; ROSSI, 1985; HOWE & SUBERKROPP, 1994). Although some invertebrates can distinguish between species mycelium (ARSUFFI & SUBERKROPP, 1985), it seamed that large shredders were not able to discriminate between species when colonies are not discrete, and no top-down effect was notice (Chapter V). On the

other hand, high current velocity seemed to stimulate fungal sporulation (Chapter V), probably due to increasing number of conidiophores per unit area.

Sporulation by aquatic hyphomycetes associated with balsa veneers was also stimulated by high nitrogen concentration in water (Chapter VI). This was expected as balsa veneers is a nutrient poor substrate (GULIS & SUBERKROPP, 2003; STELZER *et al*., 2003) and corroborates field results (Chapter III).

Generally, decomposition rates of poor quality litter were sensitive to low to moderate nutrient enrichment of water and proved to be useful evaluating the functional integrity of streams. Sporulation rates by aquatic hyphomycetes were the most sensitive microbial parameter to increase in dissolved nutrients. Fungal community structure was also sensitive to changes in riparian vegetation composition although this was not reflected into different decomposition rates which denotes some functional redundancy among decomposers.

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Verónica Ferreira

20, Junho, 2006

There has been a lot said about the sacredness of our land which is our body; and the values of our culture which is our soul; but water is the blood of our tribes, and if its life-giving flow is stopped, or it is polluted, all else will die and the many thousands of years of our communal existence will come to an end.

Frank Tenorio

In headwater streams, which are the majority of streams in catchments, the primary source of carbon and energy for aquatic food webs is allochthonous organic matter provided by riparian trees. The rate at which litter is decomposed is known to depend on several biotic (e.g. litter quality, shredders abundance) and abiotic (e.g. temperature, current, dissolved nutrients) factors which affect mass loss directly through leaching and physical fragmentation or indirectly through their effects on microbes and invertebrates, that are the major players in this ecosystem level process. As litter decomposition is an integrative process of both biological and environmental parameters, along time and through different organizational levels, it as been proposed to be used as a tool to assess streams functional integrity. The decomposition rates of nutrient poor substrates (e.g. oak leaves and balsa veneers), incubated in coarse mesh bags, in 5 oligotrophic and 5 similar nutrient enriched streams (paired design; Chapter II) and along a small forested stream where an artificial nitrate gradient was created (Chapter III), were useful assessing nutrient enrichment of streams as they were stimulated by an increase in dissolved nutrients (Chapter II and III). This stimulation of mass loss was mediated by increasing fungal (Chapter II and III) and invertebrate activity (Chapter II). Fungal sporulation was the most sensitive fungal parameter to the increase in dissolved nutrients (Chapters II, III and VI) and was also stimulated by increased current velocity (Chapter V). However, for litter decomposition rates to be used as an assessment tool of anthropogenic stress, the effect of natural inter-habitat variability must be ruled out. Mass loss of the soft alder leaves was not affect by low to moderate current velocity in either artificial channels or a 4th order stream in summer; however, in autumn mass loss was higher in high current sites, probably due to the presence of suspended sediments resulting from rains (Chapter I). Also, litter decomposition was mostly affected by shredder presence in artificial channels (Chapter I). To overcome this natural inter-habitat variability in litter decomposition one must be careful and allocate litter bags in similar sites within a stream and between streams. Besides the cultural nutrient enrichment of stream water, the other major anthropogenic impact to freshwaters is the substitution of native deciduous forest by eucalyptus monocultures. Changes in the riparian vegetation affected the fungal communities composition in Portugal more than in Spain (Chapter IV), which could be explained by the wetter climate in Spain which allows for the development of a understory of deciduous trees in eucalyptus plantations that results in the increase of litter diversity in streams. Nevertheless, decomposition of litter was similar between streams running through deciduous forests and eucalyptus plantations, in both countries, which denote some functional redundancy among decomposers (Chapter IV).