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# Assessing structural and functional ecosystem condition using leaf breakdown: studies on a polluted river

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Running title: Leaf breakdown and decomposer assemblages in a polluted river

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## SUMMARY

1. Leaf breakdown rates of *Alnus glutinosa* were determined and the structure of decomposer assemblages associated with leaves were analysed to assess the effect of pollution on the ecological condition of the Ave River (Northwest Portugal).

2. The increase in organic and inorganic nutrients was associated with an increase in the density and a decrease in the richness of macroinvertebrates, a dramatic decline in the conidial production of aquatic hyphomycetes, but no major change in the richness of aquatic hyphomycetes.

3. The nutrient enrichment toward downstream was correlated with a significant acceleration in leaf breakdown rates.

4. The degree of functional impairment assessed by the ratio of leaf breakdown rates in coarse-mesh and fine-mesh bags was in accordance with the gradient of pollution defined by two biotic indices.

5. This study supports the contention that leaf breakdown experiments are a valuable tool to assess the effect of pollution on the ecological condition of rivers.

## Introduction

The concept of ecological integrity in streams can be envisaged as structural and functional integrity and, therefore, the assessment of a stream condition should include measurements of structural biological parameters and the analysis of ecosystem level-processes (Gessner & Chauvet 2002). Leaf breakdown is an integrative process since it links riparian vegetation, microbial and invertebrate activities as well as physical and chemical features of the stream (Benfield 1996, Gessner, Dobson & Chauvet 1999). Several studies demonstrate that anthropogenic stress affects leaf breakdown rates (see Gessner & Chauvet 2002 and references therein). Some authors find that nutrient enrichment stimulates leaf breakdown (e.g. Robinson & Gessner 2000, Pascoal, Cássio & Gomes 2001), while other working groups have demonstrated that this is not always the case. In a Hong Kong stream, the presence of organic pollution led to an increase in leaf breakdown rates in summer and a decrease in winter (Au, Hodgkiss & Vrijmoed 1992a), and no effect of pollution was found in an Indian river (Raviraja, Sridhar & Bärlocher 1998). Leaf breakdown rates were lowered by mine effluent discharge (Bermingham, Maltby & Cooke 1996) and they were negatively correlated with the concentration of dissolved zinc in stream water (Niyogi, Lewis & McKnight 2001). However, high values for leaf breakdown rates were found in a moderately heavy metal polluted stream, which was explained by the presence of an adapted fungal community and high N and P concentrations in the stream water (Sridhar *et al.* 2001).

Three groups of organisms are recognised to be involved in leaf breakdown in aquatic ecosystems, namely invertebrate shredders, fungi and bacteria (Bärlocher 1992, Gessner *et al.* 1999). There is evidence that fungi, particularly aquatic hyphomycetes, dominate microbial leaf breakdown and condition the leaves, increasing their palatability for invertebrate shredders (Suberkropp 1998a). The relative importance of shredders, fungi and bacteria, and the factors controlling possible shifts in dominance are not clear (Hieber & Gessner 2002). The role of microorganisms and invertebrates in polluted streams may be altered if they respond differently to the imposed stress. It is currently recognised that macroinvertebrate communities are differentially sensitive to various types of pollutants and are capable of a graded response (Metcalfe-Smith 1996). Major data analysis for assessing water quality includes the use of biotic indices, diversity measures and multivariate analysis, widely applied to the benthic macroinvertebrate communities. Aquatic hyphomycetes are generally considered to be more abundant in streams with low level of pollution (Bärlocher 1992). Some studies, in organically polluted rivers, point to a loss of aquatic hyphomycete species and a decrease in conidial production associated with decomposing leaves (Au, Hodgkiss & Vrijmoed 1992b, Raviraja *et al.* 1998). In heavy metal polluted streams, a decline in the number of hyphomycete species (Bermingham *et al.* 1996) and a reduction in conidial production (Sridhar *et al.* 2001) were found. However, according to other studies, the community structure of aquatic hyphomycetes is not affected by effluents from a sewage treatment plant (e.g. Suberkropp *et al.* 1988) and a substantial number of hyphomycete species has been reported in some extremely polluted waters (Krauss *et al.* 2001, Sridhar *et al.* 2000).

In the current study, leaf breakdown rates of *Alnus glutinosa* (alder) were determined and the structure of the invertebrate and aquatic hyphomycete assemblages associated with leaves were analysed to assess the effect of pollution in the ecological condition of a lowland river located in the Northwest Portugal, in a region of high urban and industrial pressure.

## Materials and methods

### *Study area*

The Ave River is located in Northwest Portugal in a region of high demographic density and several industrial activities that have developed over the last 30 years, mainly textile, cutlery and metalworking industries. An integrated scheme for the recovery of this river is being implemented and most pollution sources are supposed to be linked to wastewater treatment stations. Six sampling sites were selected along a 30 km stretch of the river (Fig. 1). Stream order varied from 3 to 5 along the study stretch. The riparian forest in the Ave River catchment has been severely affected by high levels of human activity at the riverbanks and the presence of exotic riparian species such as *Acacia* sp. Nevertheless, there were only minor changes in the riparian vegetation among the sampling sites. The floristic inventory showed a dominance of *Alnus glutinosa* (L.) Gaertner in a phytosociological association characterised as *Senecio bayonnensis-Alnetum glutinosae* (Botelho 2001).

### *Field procedures*

This study started in November 1999 and ran over five weeks. Leaves of *A. glutinosa* were collected just before abscission, stored air dried, weighed into 6 g-groups and placed in fine-mesh (0.5 mm mesh) and coarse-mesh (10 mm mesh) bags (16 x 20 cm). A total of 108 bags of each mesh size were sealed and distributed at the sampling sites. Triplicate fine-mesh and coarse-mesh bags were retrieved from each sampling site weekly over the period of study. In addition, three bags of both mesh sizes were randomly retrieved after thirty minutes of immersion to determine the initial leaf mass.

### *Physical, chemical and microbial analyses of stream water*

During the study period, water samples were collected into sterile glass bottles, transported in a cold box (4 °C) and analysed within 24 h. A HACH DR/2000 photometer was used for the following analyses: quantification of chemical oxygen demand (COD) by dichromate reactor digestion method (measurable range: 0-40 mg $L^{-1}$), determination of nitrate concentration by cadmium reduction method (measurable range: 0-30 mg $L^{-1}$) and determination of ammonium concentration by the Nessler method (measurable range: 0.025-3.25 mg $L^{-1}$). The colony-forming units (CFU) of total heterotrophs, total and faecal coliforms were quantified according to standard methods (APHA 1998).

### *Leaf mass loss*

The leaves were removed from the bags, rinsed with deionized water to remove the sediments and adhering invertebrates. Subsamples of the leaf material were used to induce fungal sporulation and the remaining leaf material was dried at 60 °C to constant mass (72±24 h) and weighed to the nearest 0.01 g.

### *Macroinvertebrate and fungal survey*

The leaves in coarse-mesh bags were rinsed into a 400 μm-mesh screen to retain the associated macroinvertebrates, which were sorted and preserved in ethanol (70 %, v/v) until identification and counting. Macroinvertebrates were identified to the genus or family, except for Oligochaeta that were not identified beyond the class level. Taxa were assigned as shredders according to Merritt & Cummins (1996). Two biotic indices, namely the Belgian Biotic Index (BBI – De Pauw & Vanhooren 1983) and the Biological Monitoring Working Party index, adapted to the Iberian Peninsula (BMWP′ – Alba-Tercedor & Sánchez-Ortega 1988, Rico *et al.* 1992) were calculated. In addition, both the relative number of Oligochaeta (% O) and of Ephemeroptera + Plecoptera + Trichoptera (% EPT) were calculated.

Fungal sporulation was induced by aeration of 15 leaf discs (12 mm diameter) from each fine-mesh bag in 40 ml of filtered stream water (0.22 μm pore size membrane) for 48±4 h at 18 °C. The suspension was filtered through a membrane (Millipore, 5 μm pore size) and the spores retained were stained with cotton blue in lactic acid, identified and counted. Sporulation rates were calculated as number of conidia released per g (dry mass) of decomposing leaves per day.

The macroinvertebrate and fungal communities were also analysed in terms of taxa richness.

### *Statistical analysis*

Rates of leaf breakdown were obtained by fitting the percentage of dry mass remaining to the exponential model $W_t = W_o \cdot e^{-kt}$ (Petersen & Cummins 1974), where k is the exponential breakdown coefficient, $W_t$ is the leaf dry mass remaining after time t from the initial amount $W_o$. Regression lines (ln transformed data) were compared by analysis of covariance (ANCOVA) followed by Tukey´s HSD test (Zar 1996), using the statistical package Prism 3.0 for Macintosh (GraphPad software Inc., San Diego).

Ordination of the spatial gradient according to the stream water variables was done using Principal Component Analysis (PCA) after standardisation of the variables (Legendre & Legendre 1998). The distribution of macroinvertebrates and fungi associated with leaf bags was analysed by Correspondence Analysis (CA – Legendre & Legendre 1998). Analyses were based on either the average number of the macroinvertebrates collected from leaves in coarse-mesh bags at each site during the whole study period, or the average value of sporulation rates of the aquatic hyphomycetes associated with leaves in fine-mesh bags at each site during the first three weeks of the study. Both PCA and CA were performed using the statistical package ADE-4 for Macintosh (Thioulouse *et al.*1997).

Spearman rank correlation was used to examine the relationship between stream water variables, leaf breakdown rates, macroinvertebrate densities and richness, and aquatic hyphomycete sporulation rates and richness. Linear regression analysis was used to investigate the effect of stream water variables described by PCA scores on leaf breakdown rates. Correlations and regressions were done using the statistical package Statview 5.0 for Macintosh (SAS Institute Inc., North Carolina).

## Results

### *Physical, chemical and microbial analyses of stream water*

Temperature was similar at the different sampling sites (range 12.4 to 13.7 ºC). Other physical, chemical and microbial characteristics of stream water are presented in Table 1. The average values of pH varied from 6.6 to 7.3. Conductivity and the chloride concentration increased about 7-fold and 18-fold, respectively, between L1 and L7. COD and the concentration of nutrients, such as ammonium, nitrates and phosphates, were high and increased from upstream to downstream. At all sampling sites, the total number of heterotrophic microbes was high and reached 1.4 x $10^6$ CFU $ml^{-1}$ at L7, the most downstream site. This site also exhibited the highest values for total and faecal coliform populations, although similar values for faecal coliforms were found at L5. The PCA ordination of the physical, chemical and microbial variables (Table 1) showed that variables indicative of a pollution gradient were positively correlated with the first PC axis (Fig. 2). The second PC axis seemed to separate inorganic nutrients from organic load. The PCA ordination of the sampling sites indicated three groups: i) L1 and L2, ii) L3, L4 and L5, and iii) L7 (Fig. 2).

### *Macroinvertebrate assemblages associated with decomposing alder leaves*

The density of macroinvertebrates associated with leaves in coarse-mesh bags increased from upstream to downstream (Fig. 3a). Significant correlations were noted between macroinvertebrate density and the concentration of nitrates (r=0.89, p=0.04), phosphates (r=0.97, p=0.04), ammonium (r=0.77, p=0.05) and conductivity (r=0.87, p=0.05) in the stream water. Macroinvertebrate richness was low at all sampling sites (Fig. 3a). The highest richness was found at L1 (20 taxa) and the lowest at L3 (7 taxa). No significant correlations were found between richness and the environmental variables tested here.

The BBI index revealed slightly polluted water at L1 (index 8) and moderately polluted water (index 5 to 6) at all downstream sampling sites (Fig. 3b). Similarly, BMWP′ (Fig. 3b) indicated some disturbance at L1, and decreased water quality at downstream sites: polluted (L2), seriously polluted (from L3 to L5) and polluted (L7). Poor water quality was also revealed by the low percentage of EPT taxa (Fig. 3c). The value was highest at L1, corresponding to about 20 % of all individuals, and declined at L2 (7 %). Below L2, EPT taxa were only found at L4 and L7 at low frequencies (< 1 %). Shredders were rare at L1 and L7, and absent at the other sampling sites (data not shown). Apart from L1 and L2, which exhibited percentages of Oligochaeta less than 10 % (Fig. 3c), all sites exhibited extremely high values, particularly from L3 (69 %) to L5 (82 %).

The distribution of the macroinvertebrates colonising leaf bags along the sampling sites by Correspondence Analysis is shown in Fig. 4. Factor 1 explained 41 % of the total variance and clearly distinguished L1 and L2 from all the other sites. The taxa responsible for the separation along factor 1 were Athericidae, Baetidae, Leptophlebiidae, Leuctridae, Polycentropodidae and Platycnemidae. These taxa exhibited higher relative and absolute contributions to factor 1 and they were mainly associated with L1 and into a lesser extent with L2 (data not shown). Factor 2 explained 26 % of the total variance and mainly separated L7 from L3, L4 and L5. Analysis of both absolute and relative contributions (data not shown) also suggested a strong association of Ancylidae, Dugesiidae, Lymnaeidae, Bythinellidae and Calopterygidae with L7, while Erpobdellidae and Oligochaeta were strongly associated with L3, L4 and L5. The CA ordination of the sampling sites indicated three groups: i) L1 and L2, ii) L3, L4 and L5, and iii) L7 (Fig. 4).

### *Aquatic hyphomycete assemblages associated with decomposing alder leaves*

Aquatic hyphomycetes exhibited peaks of sporulation between the first and the third week of leaf immersion in the Ave River, depending on the sampling site (data not shown). Average values of sporulation rates during the first three weeks of leaf decomposition are shown in Fig. 5. The highest value was observed at L1 (2140 conidia $mg^{-1}$ leaf dry mass $d^{-1}$), which decreased about 100-fold at L5 and increased slightly at L7 (187 conidia $mg^{-1}$ leaf dry mass $d^{-1}$). Sporulation rates were negatively correlated with the concentrations of ammonium (r=-0.90, p=0.05) and chloride (r=-0.97, p=0.05) in the stream water. Fungal richness ranged from 18 to 23 taxa and the highest value was found at L7.

CA ordination of the aquatic hyphomycete taxa colonising alder leaves in fine-mesh bags is shown in Fig. 6. Factor 1 explained 51 % of the total variance and separated L1 from the other sampling sites. Factor 3 explained 17 % of the total variance and seemed to separate L3 from the other sampling sites. Analysis of absolute and relative contributions (data not shown) suggested that Sigmoid 1, *Alatospora acuminata* and *Tetrachaetum elegans* were clearly associated with L1, while *Clavariopsis aquatica*, *Clavatospora longibrachiata*, *Heliscella stellata* and *Tricladium splendens* were mainly related to L3. In addition, *C. longibrachiata* made up more than 50 % of the total number of conidia released from submerged leaves at

L3. *Flagellospora curta* and *Heliscus lugdunensis* also exhibited high absolute and relative contributions (data not shown) and were strongly related to L5 and L7. In contrast, *Anguillospora filiformis* and *Articulospora tetracladia* were widely distributed along the study stretch, and thus did not contribute to the ordination of the sampling sites. The CA ordination of the sampling sites indicated three groups: i) L1, ii) L2, L5 and L7, and iii) L3 (Fig. 6).

### *Breakdown rates of alder leaves*

Leaf breakdown rates were high (Petersen and Cummins 1974) and increased from upstream to downstream (Table 2). All sampling sites, except L1, exhibited significantly faster leaf breakdown in coarse-mesh than in fine-mesh bags (ANCOVA, $F=4.15$, $p<0.05$). Significant differences were found among the sampling sites in both fine-mesh (ANCOVA, $F=2.42$, $p=0.04$) and coarse-mesh (ANCOVA, $F=8.61$, $p<0.0001$) bags. Leaf breakdown was more pronounced in coarse-mesh bags at L7 ($k_c=0.0369$ $d^{-1}$) when compared to that measured at L1 and L2, but no differences were found between L7, L3, L4 and L5 (Table 2). In fine-mesh bags, L7 ($k_f=0.0195$ $d^{-1}$) exhibited significantly faster leaf breakdown than L1, L2 and L3, and leaf breakdown rates at L4 and L5 were not significantly different from those measured at all the other sites.

Significant linear relationships were found between the gradient of pollution, as defined by scores of the first PC axis in Fig. 2, and leaf breakdown rates in both coarse-mesh ($r=0.96$, $p=0.002$) and fine-mesh ($r=0.92$, $p=0.01$) bags (Fig. 7). In addition, leaf breakdown rates in fine-mesh bags were significantly correlated with nitrates ($r=0.77$, $p=0.05$), but not with aquatic hyphomycete attributes. Leaf breakdown rates in coarse-mesh bags were correlated with ammonium (r=0.95, p=0.03), nitrates (r=0.94, p=0.04), phosphates (r=0.88, p=0.05) and macroinvertebrate density (r=0.77, p=0.05).

## Discussion

### *Decomposer assemblages and structural condition of the Ave River*

In the reach under study, the concentration of organic and inorganic nutrients was high and increased along the longitudinal gradient of the river. From upstream to downstream, sporulation rates of aquatic hyphomycetes declined and were negatively associated with ammonium and chloride concentrations in the stream water. A decline in the number of aquatic hyphomycete species and in the spore production has been observed in streams polluted with either organic compounds (Au *et al.* 1992b, Raviraja *et al.* 1998) or heavy metals (Sridhar *et al.* 2001). In the present study, the total number of aquatic hyphomycete species varied little (18-23 taxa) and was comparable with those reported from alder leaves by Bärlocher, Canhoto & Graça (1995) in Central Portugal (22-24 taxa) and by Chauvet *et al.* (1997) in Northern Spain (15-17 taxa). However, CA ordination indicated community changes on either the identity or the relative proportion of hyphomycete species along the Ave River. The density of macroinvertebrates in alder leaves was correlated with nitrate and phosphate concentrations in the stream water. Higher density of invertebrates, in both alder and eucalyptus leaves, was also found at nutrient enriched downstream sites in Northern Spain (Basaguren & Pozo 1994). Ordination of the sampling sites based on either the environmental variables or the macroinvertebrate taxa discriminated the same groups, namely i) L1 and L2, ii) L3, L4 and L5, and iii)

L7. Nevertheless, multivariate approaches cannot directly show whether ecosystem conditions are improving or deteriorating (Cao, Bark & Williams 1996).

Biotic indices, such as BBI and BMWP', developed on benthic macroinvertebrate communities are designed for water quality assessment using information on species tolerance to pollution (Metcalfe-Smith 1996). Previous studies demonstrate that the degree of pollution as evaluated by biotic indices can depend on both the sampling method and the biotic index used (Pascoal *et al.* 2001). Graça, Ferreira & Coimbra (2001) recovered essentially the same invertebrate taxa using either alder leaf bags or hand net, and these sampling methods were effective in discriminating water quality in a stream impacted by sewage effluents in the Northwest of Portugal (Pascoal *et al.* 2001). BMWP' and BBI were good indicators of water pollution, being the first index more sensitive to changes in water quality (Pascoal *et al.* 2001). Similar results were found in the Ave River, and BMWP' classified L1 as having some disturbance, L2 and L7 as polluted, and L3, L4 and L5 as seriously polluted. Ephemeroptera, Plecoptera and Trichoptera include sensitive taxa to organic pollution (Resh & Jackson 1993), while Oligochaeta include several tolerant taxa (Merritt & Cummins 1996). Below L2, the low frequencies of EPT taxa and the dominance of Oligochaeta corroborate the poor water quality at downstream sites.

### *Leaf breakdown and functional condition of the Ave River*

Breakdown rates of alder leaves were high and varied from 0.0113 to 0.0195 $d^{-1}$ and from 0.0170 to 0.0369 $d^{-1}$ in fine-mesh and coarse-mesh bags, respectively. These values are within the range of those reported for alder leaves in Portuguese

(Abelho 1999, Graça *et al.* 2001), Spanish (Pozo *et al.* 1998), French (Gessner & Chauvet 1994, Fabre & Chauvet 1998) and German (Hieber & Gessner 2002) streams, in the same season.

In this study, leaf breakdown was faster at the most nutrient enriched sites as found by other authors (Meyer & Johnson 1983, Suberkropp & Chauvet 1995, Pearson & Connolly 2000, Pascoal *et al.* 2001). Leaf breakdown can be stimulated by phosphate or both phosphate and nitrate (Grattan & Suberkropp 2001) and differences in nitrate concentration appeared to explain much of the variation in leaf breakdown rates and in microbial activity in streams with different water chemistry (Meyer & Johnson 1983, Suberkropp & Chauvet 1995). However, most of the research on the effect of nutrients on leaf breakdown has been conducted in streams with lower levels of nutrients (e.g. Meyer & Johnson 1983, Suberkropp & Chauvet 1995, Robinson & Gessner 2000, Grattan & Suberkropp 2001**)** when compared to those found in this study. In the Ave River, the stress gradient described by PCA revealed to be a reliable predictor of leaf breakdown rates in both fine-mesh and coarse-mesh bags. Leaf breakdown rates in fine-mesh bags were mainly correlated with nitrate concentrations and rates in coarse-mesh bags were correlated with ammonium, nitrate and phosphate concentrations, which could suggest a different influence of N and P on microbial decomposing activity depending on the presence or absence of feeding invertebrates.

Significant correlations between leaf breakdown rate and maximum of fungal biomass or conidial production have been found (Gessner & Chauvet 1994, Maharning & Bärlocher 1996). However, in an organically polluted river, the decline in spore production was not accompanied by a decrease in leaf breakdown rates

(Raviraja *et al.* 1998). In the Ave River, leaf breakdown rates were not significantly correlated with fungal sporulation and at the most downstream site, which exhibited the highest leaf breakdown rate, low conidium production was found. Several hypotheses can explain these findings. First, higher values for spore production could have occurred outside the sampling dates. Second, sporulation is more sensitive to water chemistry than growth of aquatic hyphomycetes (e.g., Suberkropp & Chauvet 1995, Suberkropp 1998b), and the reduction in sporulation rates is not necessarily proportional to the decrease in fungal biomass and/or enzymatic decomposing activity. Finally, although fungi are considered to contribute greater to leaf mass loss than heterotrophic bacteria (Baldy, Gessner & Chauvet 1995, Weyers & Suberkropp 1996, Hieber & Gessner 2002), an enhanced contribution of bacteria for the overall process could have occurred at the most polluted sites.

Apart from L1, leaf breakdown rates were higher in coarse-mesh bags and were correlated with the density of macroinvertebrates. Shredders were rare or even absent at the majority of the sampling sites and thus, could not have been responsible for the faster leaf breakdown observed at the most nutrient enriched sites. Similar results were found at sites affected by sewage effluents (Pascoal *et al.* 2001). Except perhaps for predators, all the other invertebrates can, to various extents, behave as detritivores (Graça 2001). A high number of oligochaetes was frequently observed inside the leaf matrix after the third week of leaf immersion in the Ave River, mainly below L2. These invertebrates, which feed on fine particulate organic matter, use leaf litter as habitat where they may enhance leaf breakdown by their movement and feeding activity (Chauvet, Giani & Gessner 1993).

Gessner & Chauvet (2002) proposed that the ratio of leaf breakdown rates in coarse-mesh bags to rates in fine-mesh bags could provide more powerful information to assess functional stream integrity than absolute values of breakdown rates, because changes in the ratio would indicate changes in the relative contribution of microorganisms and invertebrates. The ratio of leaf breakdown rates in coarse-mesh and fine-mesh bags (Table 3) revealed that apart from L1 (score 2), ecosystem functioning in the Ave River was compromised at all sampling sites, particularly at L3 (score 0). The better river functioning at L1 and the compromised functional condition at downstream sites were in agreement with data from biotic indices. Furthermore, CA ordination of aquatic hyphomycetes separated L3 from either L1 or the remaining sampling sites, suggesting shifts in the structure of the fungal assemblages associated with changes in the functional condition of the Ave River.

In summary, structural and functional attributes in the Ave River responded to the presence of pollution as follows: i) an increase in the density and a decrease in the richness of macroinvertebrate taxa, ii) a strong decline in conidial production, but no major change in the richness of aquatic hyphomycete taxa and iii) a stimulation of leaf breakdown rates. In addition, the degree of functional impairment assessed by the ratio of leaf breakdown rates in coarse-mesh and fine-mesh bags was in accordance with the gradient of pollution defined by two biotic indices. Therefore, these results support the contention that studies of leaf breakdown are valuable tools to assess the effect of pollution on the ecological condition of rivers.

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