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Stimulation of leaf litter decomposition and associated fungi and invertebrates by moderate eutrophication: implications for stream assessment

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SUMMARY

1. We investigated the effect of moderate eutrophication on leaf litter decomposition and associated invertebrates in five reference and five eutrophied streams in central Portugal. Fungal parameters and litter N and P dynamics were followed in one pair of streams. Benthic invertebrate parameters that are considered useful in bioassessment were estimated in all streams. Finally, we evaluated the utility of decomposition as a tool to assess stream ecosystem functional integrity.
2. Decomposition of alder and oak leaves in coarse mesh bags was on average 2.3–2.7× faster in eutrophied than in reference streams. This was attributed to stimulation of fungal activity (fungal biomass accrual and sporulation of aquatic hyphomycetes) by dissolved nutrients. These effects were more pronounced for oak litter (lower quality substrate) than alder. N content of leaf litter did not differ between stream types, while P accrual was higher in the eutrophied than in the reference stream. Total invertebrate abundances and richness associated with oak litter, but not with alder, were higher in eutrophied streams.
3. We found only positive correlations between stream nutrients (DIN and SRP) and leaf litter decomposition rates in both fine and coarse mesh bags, associated sporulation rates of aquatic hyphomycetes and, in some cases, total invertebrate abundances and richness.
4. Some metrics based on benthic invertebrate community data (e.g. % shredders, % shredder taxa) were significantly lower in eutrophied than in reference streams, whereas the IBMWP index that is specifically designed for the Iberian peninsula classified all 10 streams in the highest possible class as having 'very good' ecological conditions.
5. Leaf litter decomposition was sufficiently sensitive to respond to low levels of eutrophication and could be a useful functional measure to complement assessment programmes based on structural parameters.

Keywords: nutrients, nitrogen, phosphorus, aquatic hyphomycetes, functional parameters

Introduction

Low-order forested streams, where light limitation restricts primary production, rely upon the input of organic matter from the riparian zone to fuel in-stream processes (Vannote *et al.*, 1980). Leaves and wood are the main sources of carbon and energy in these streams (e.g. Kaushik & Hynes, 1971; Webster & Meyer, 1997) and decomposition of this organic matter is the key ecosystem-level process integrating the activities of both microbial decomposers (primarily fungi) and aquatic invertebrates (Gessner & Chauvet, 1994; Suberkropp, 1998b; Graça, 2001; Hieber & Gessner, 2002).

Eutrophication of streams and rivers is a worldwide problem that has many consequences for both aquatic

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communities and ecosystem processes (e.g. Vitousek *et al.*, 1997; Nijboer & Verdonschot, 2004). Several previous studies (Elwood *et al.*, 1981; Gulis & Suberkropp, 2003b; Gulis *et al.*, 2004; Benstead *et al.*, 2005; Ferreira, Gulis & Graça, 2006b; Greenwood *et al.*, 2006) have shown that experimental whole-stream nutrient enrichments of small headwater streams generally accelerate leaf litter and wood decomposition and stimulate associated microbial activity. However, reported effects of cultural eutrophication on decomposition and microbial parameters vary (Suberkropp *et al.*, 1988; Young, Huryn & Townsend, 1994; Molinero, Pozo & González, 1996; Huryn *et al.*, 2002; Niyogi, Simon & Townsend, 2003; Pascoal *et al.*, 2003; Lecerf *et al.*, 2006). It is more difficult to predict the response of stream biota and processes to anthropogenic eutrophication as the increase in nutrients is often accompanied by other pollutants with variable effects on stream communities and ecosystem function. Sedimentation or low oxygen concentration in stream water (e.g. Au, Hodgkiss & Vrijmoed, 1992a,b; Raviraja, Sridhar & Bärlocher, 1998; Niyogi *et al.*, 2003) or even toxic ammonium levels (Lecerf *et al.*, 2006) may complicate the matter further. We argue that understanding the effects of actual eutrophication, as opposed to that in the experimentally manipulated streams, would be advanced by greater focus on low to moderate levels of eutrophication. First, this approach would help to separate the effects of nutrients from other causes. Secondly, we need better tools to diagnose the impact of low chronic levels of pollution on communities and ecosystem functioning.

Current approaches to assess river health are primarily based on structural parameters, such as species richness, identities and abundances. Good examples are biotic indices based on benthic macroinvertebrates or periphyton, especially diatoms (e.g. Metcalfe-Smith, 1994; Barbour *et al.*, 1999; Hill *et al.*, 2000). Alternative or complementary approaches based on functional parameters should monitor key ecosystem-level processes such as community metabolism or decomposition (e.g. Bunn, Davies & Mosisch, 1999; Bunn & Davies, 2000; Gessner & Chauvet, 2002; Young, Townsend & Matthaei, 2004), which provide integrative measures of ecosystem health. For example, plant litter decomposition in temperate regions is governed by the activities of bacteria, fungi and invertebrates that may respond differently to anthropogenic stress. As decomposition of allochthonous organic matter is the major energy pathway in forested streams, it could be a promising tool to assess stream ecosystem functioning (Gessner & Chauvet, 2002; Pascoal *et al.*, 2003). If we are to understand the mechanisms underlying changes in ecosystem functioning, we also have to identify the key parameters (e.g. changes in microbial or shredder community structure, biomass and activity) that lead to changes in decomposition rates.

Thus, the main objectives of this study were to test (1) whether the results of whole-stream nutrient enrichment experiments are transferable to streams with moderate nutrient concentrations due to human activity and (2) whether decomposition could be a useful tool to indicate changes in ecosystem functioning due to eutrophication. To address these questions we determined leaf litter decomposition rates and associated fungal and invertebrate parameters in paired reference and eutrophied streams. Two types of leaf litter contrasting in nutrient content that may respond differently to nutrients in water (e.g. Stelzer, Heffernan & Likens, 2003; Ferreira *et al.*, 2006b) were used. Both fine and coarse mesh litter bags were used, to distinguish the effects of eutrophication on the microbes and macroinvertebrates that both control decomposition. Benthic invertebrate parameters that are considered useful in bioassessment were also calculated and their performance compared with that of the decomposition assay.

Materials and methods

Study sites

Experiments were conducted in 10 streams of the Vouga and Mondego river basins in central Portugal (Table 1). All streams had similar geology (siliceous bedrock) and physico-chemical characteristics (except dissolved inorganic nutrients, see below) (Table 1). All were second to fourth order (1.5–5 m wide) softwater streams (alkalinity <9 mg $CaCO_3$ L^{-1}) running through native deciduous forests or at least had deciduous trees in the riparian corridor as in case of some eutrophied streams. Riparian vegetation was dominated by oak (*Quercus robur* L.), chestnut (*Castanea sativa* Mill.) and in some streams alder [*Alnus glutinosa* (L.) Gaertn.]. Five reference (SRP below 20 μg L^{-1}) and five eutrophied streams (SRP above

Table 1 Some physico-chemical characteristics of streams during decomposition experiments (25 November 2002–10 April 2003; mean ± SD, n = 4–7)

Stream	Stream pair	Latitude (N)	Longitude (W)	Altitude (m a.s.l.)	Substrate	Water temperature (°C)	pH	Conductivity (µS cm^{-1})	NH_4-N (µg L^{-1})	NO_3-N (µg L^{-1})	SRP (µg L^{-1})
Reference streams											
Candal 1	C1	40°04′44″	8°12′10″	620	CP	10.7 ± 1.2	6.5 ± 0.2	25 ± 1	BDL-117	72 ± 14	6 ± 1
Sardeira	S2	40°05′21″	8°12′06″	520	CP	10.6 ± 1.2	6.5 ± 0.4	30 ± 3	BDL-458	76 ± 16	5 ± 1
Agueda	S3	40°35′44″	8°10′30″	805	CP	9.5 ± 1.0	5.8 ± 0.1	25 ± 1	BDL	483 ± 132	3 ± 1
Agadao	S4	40°31′59″	8°13′53″	570	PS	11.5 ± 1.1	5.8 ± 0.1	27 ± 3	BDL	114 ± 72	4 ± 1
Tojosa	S5	40°31′29″	8°11′08″	255	BCPS	10.3 ± 1.2	6.4 ± 0.2	27 ± 1	BDL	42 ± 17	16 ± 4
Eutrophied streams											
Casteloes	C1	40°32′01″	8°09′15″	210	CPS	11.0 ± 0.9	6.4 ± 0.2	54 ± 2	BDL-13	1127 ± 173	29 ± 5
Muceres	S2	40°32′05″	8°09′31″	215	CPS	10.8 ± 0.9	6.4 ± 0.2	31 ± 2	BDL	216 ± 160	25 ± 4
Caramulo 1	S3	40°34′12″	8°09′04″	455	BCP	10.7 ± 0.6	6.5 ± 0.4	59 ± 6	BDL-209	2153 ± 340	26 ± 19
Caramulo 2	S4	40°34′04″	8°09′03″	430	PS	11.0 ± 1.0	6.7 ± 0.2	82 ± 12	BDL	2995 ± 665	28 ± 7
Criz	S5	40°32′28″	8°07′18″	245	CPS	11.0 ± 1.1	6.6 ± 0.2	56 ± 5	120 ± 74	1340 ± 407	56 ± 43

B, boulders; C, cobbles; P, pebbles; S, sand; BDL, below detection limit (10 µg L^{-1} for NH_4-N).

20 µg L^{-1} that also coincided with elevated DIN) were selected. We paired streams based on their stream bottom substrate, width/order and riparian vegetation before starting the decomposition experiments. Paired streams differed mainly in concentrations of both DIN and SRP in water with all other variables being similar (Table 1). Leaf litter decomposition rates and macroinvertebrate parameters were estimated in all streams while a suite of microbial parameters was followed in complete time series streams only (pair C1, Table 1, see below).

Water parameters

Water temperature in all streams during the experiment was continuously monitored with ACR Smart-Button (ACR Systems Inc., Surrey, BC, Canada) or Optic StowAway (Onset Computer Corp., Pocasset, MA, U.S.A.) temperature loggers (25 November 2002–10 April 2003). Stream water conductivity (WTW LF 330, Weilheim, Germany), pH (JENWAY 3310, Essex, U.K.) were measured and water samples for nutrient analyses were taken on each field trip during the decomposition experiments (n = 4–7). Water was passed through glass fibre filters (APFF, Millipore, Bedford, MA, U.S.A.) in the field, transported to the laboratory on ice and frozen to be later analysed for nitrate and SRP. Additionally, unfiltered water samples were used to determine ammonium concentration and alkalinity within 24 h of collection. Nitrate and ammonium were determined by ion chromatography (Dionex DX-120, Sunnyvale, CA, U.S.A.), SRP by the ascorbic acid method [American Public Health Association (APHA), 1995] and alkalinity according to the low alkalinity method (APHA, 1995).

Leaf bags and decomposition

Alder and oak leaves were collected from the same stands of trees in autumn 2002, just after abscission, and were air-dried and stored until needed. Leaves were weighed in batches of 4.5–5 g, moistened, enclosed in fine or coarse mesh bags (0.5 and 10 mm mesh size respectively) and deployed in streams on 25 November 2002 (alder) and 26–27 January 2003 (oak). Six extra bags of each leaf type were used to determine an initial dry mass to ash-free dry mass (AFDM) conversion factor.

Six bags of each leaf type and mesh size (24 total) were tied with short lines to iron bars driven into the stream bottom in each of the 10 streams in 12- to 30-cm deep riffles. Paired fine and coarse mesh bags were interspersed on the same line. Rebars were arranged in 12- to 30-cm deep riffles. Additional 64 bags were submerged in each of the complete time series streams (pair C1). All litter bags with a certain leaf type from the S series 'simplified' streams (pairs S2–S5) were retrieved simultaneously (day 26 for alder and day 57 for oak with the exceptions of eutrophied streams of pairs S4 and S5 where bags were retrieved on days 22 and 42 of the experiment, respectively, because of fast breakdown). That roughly corresponded to the time needed for 50% mass loss in reference streams that was predicted from samples in the complete time series streams (see below). Retrieved leaf bags were placed in individual zip-lock bags, brought to the laboratory on ice and frozen. Upon thawing, each sample was gently rinsed with distilled water onto a 500-μm mesh sieve to remove sediments and macroinvertebrates. Leaf material was dried at 105 °C for 24–48 h, weighed, ashed at 550 °C for 4–6 h and reweighed to determine AFDM remaining.

On each of five sampling dates, four to six bags of each leaf type and mesh size from complete time series streams (pair C1) were placed in zip-lock bags, brought to the laboratory on ice (but not frozen) and processed within 24 h as described above. In addition, leaf material from each bag was used to punch out two sets of five leaf disks each (12 mm diameter). One set of disks was frozen at −20 °C for fungal biomass determination and the other was used to induce sporulation of aquatic hyphomycetes (see below). The remaining leaf material was dried at 105 °C for 24–48 h, weighed, ground (1 mm screen, Retsch ZN 100; Haan, Germany) and subsampled for determination of nitrogen, phosphorus and ash content.

Nitrogen and phosphorus content of leaf litter

Nitrogen and phosphorus content was determined from subsamples of ground leaf material. Nitrogen was measured with a Perkin Elmer 2400 Series II CHNS/O analyzer (Boston, MA, U.S.A.). Phosphorus was determined spectrophotometrically after mixed acid digestion (15 min at 325 °C; Allen, 1989).

Fungal biomass and sporulation rate of aquatic hyphomycetes

Fungal biomass associated with leaf litter in coarse mesh bags was determined from ergosterol concentrations. Frozen sets of leaf disks were freeze-dried and ergosterol extraction carried out immediately. Lipids were extracted and saponified in alkaline methanol (8 g KOH L^{-1}) at 80 °C for 30 min, with stirring. The extract was then purified by solid phase extraction (Sep-Pak VacRC tC_{18} cartridges, Waters, Milford, MA, U.S.A.) (Gessner, 2005). Ergosterol in isopropanol extracts was quantified with HPLC by measuring absorbance at 282 nm. External ergosterol standards (Fluka, Buchs, Switzerland) were used for calibration. The HPLC system (Dionex) was equipped with a reverse phase C_{18} column (Brownlee Spheri-5 RP-18; Applied Biosystems, Foster City, CA, U.S.A.) maintained at 33 °C. 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 mg ergosterol g^{-1} fungal dry mass (Gessner & Chauvet, 1993).

To induce sporulation of aquatic hyphomycetes, sets of leaf disks from each leaf bag from complete time series streams were used. Leaf disks were incubated in 25 mL filtered stream water (glass fibre, APFF, Millipore) in 100 mL Erlenmeyer flasks on an orbital shaker at 100 rpm for 48 ± 2 h at 15 °C. Conidia suspensions were transferred into 50-mL centrifuge tubes, flasks rinsed twice, and conidia fixed with 2 mL of 37% formalin to be counted later. The corresponding disks were saved and their AFDM was determined as described above for bulk leaf material. When preparing slides for counting conidia, 100 μL of 0.5% Triton X-100 solution were added to the suspension to ensure a uniform distribution of conidia, suspension was stirred and an aliquot filtered (Millipore SMWP, 5 μm pore size). Filters were stained with cotton blue in lactic acid (0.05%) and spores were counted with a compound microscope (Leitz Diaplan, Wetzlar, Germany) at 200×.

Macroinvertebrates

Macroinvertebrates were analysed from both litter bags and benthic samples. After rinsing each litter bag sample, macroinvertebrates retained on a 500-μm sieve were collected and stored in 70% ethanol until

counted and identified. Kick-net benthic invertebrate samples were collected twice during the experimental period in all streams (2 and 4 December 2002, and 31 January and 1 February 2003 that roughly corresponded to the time of 50% mass loss in alder and oak leaf bags, respectively). Sampling was standardised by time (1 min kicking) and area (*c.* 0.3 × 1 m). On each sampling date, six subsamples from different stream microhabitats were combined. Samples were preserved with 4% formalin and later sorted, identified and counted. Identification was 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). Invertebrates were classified as shredders according to Tachet *et al*. (2000). Invertebrate data were used to calculate biotic metrics for each sampling date separately that were then averaged. This included IBMWP index (Alba-Tercedor *et al*., 2002; Jáimez-Cuéllar *et al*., 2002), which is an Iberian adaptation of the BMWP (Biological Monitoring Working Party, Armitage *et al*., 1983).

Statistical analysis

As experiments with alder and oak leaves were started at different time and because it is well known that they are two contrasting substrates with respect to breakdown rates and other parameters, all data from these substrates were analysed separately. Decomposition rates 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 we adopted a paired stream design, we used a paired *t*-test for means to compare leaf litter decomposition rates and total invertebrate abundances and richness associated with leaf litter between reference and eutrophied streams. We also run nested ANOVA on AFDM remaining data from 10 streams without taking pairing into account (streams nested within disturbance, litter bags as subsamples) to test the effect of eutrophication (Zar, 1984). Differences in decomposition rates in complete time series streams were assessed by ANCOVA (comparison of slopes) followed by Tukey's test (Zar, 1984). Nitrogen and phosphorus contents of leaf litter, fungal biomass and sporulation rates of aquatic hyphomycetes associated with leaf litter in complete time series streams were compared between streams and litter bag mesh sizes by randomised block ANOVA (time as blocking variable) separately for each type of litter. Sporulation data were $\log_{10}$ transformed before analysis. Invertebrate parameters from benthic samples were compared between reference and eutrophied streams by paired *t*-test. Relationships between inorganic dissolved nutrients in water and decomposition rates, and fungal and invertebrate parameters associated with leaf litter were explored by fitting data into both linear and Michaelis–Menten models. For this purpose, data from five additional reference streams in the same area in central Portugal that were sampled during the same time frame using exactly the same experimental protocol (Ferreira *et al*., 2006a; V. Gulis, V. Ferreira & M.A.S. Graça, unpublished data) were used. For oak litter, further data from a pilot experiment in three streams [pair C1 of this study and Portuguese deciduous stream in Ferreira *et al.* (2006a)] were used. Statistical analyses were done with Statistica 6.

Results

Decomposition

Decomposition rates of alder leaves did not differ significantly between reference and eutrophied streams for both coarse mesh bags (paired *t*-test, $P = 0.080$; nested ANOVA (streams not paired), $F_{1,8} = 3.54$, $P = 0.096$) and fine mesh bags (paired *t*-test, $P = 0.120$; nested ANOVA, $F_{1.8} = 2.37$, $P = 0.162$), even though some tendency for faster decomposition in the eutrophied streams was apparent (Fig. 1). Decomposition of oak leaves was significantly faster in the eutrophied streams in comparison with the reference streams in both coarse mesh bags (paired *t*-test, $P = 0.017$; nested ANOVA, $F_{1.8} = 10.54$, $P = 0.012$) and fine mesh bags (paired *t*-test, $P = 0.032$; nested ANOVA, $F_{1.8} = 6.19$, $P = 0.038$) (Fig. 1). The typical differences in leaf litter mass loss through time for two leaf species and two mesh sizes are shown for the stream pair C1 (Fig. 2a,b). The overall effects of both mesh size (ANCOVA, $P < 0.001$ for alder and oak) and stream type (eutrophication) (ANCOVA, $P < 0.001$ for alder, $P = 0.036$ for oak) on mass loss were significant with decomposition being faster in coarse mesh bags and in the eutrophied stream. However, Tukey's test indicated that only decomposition in

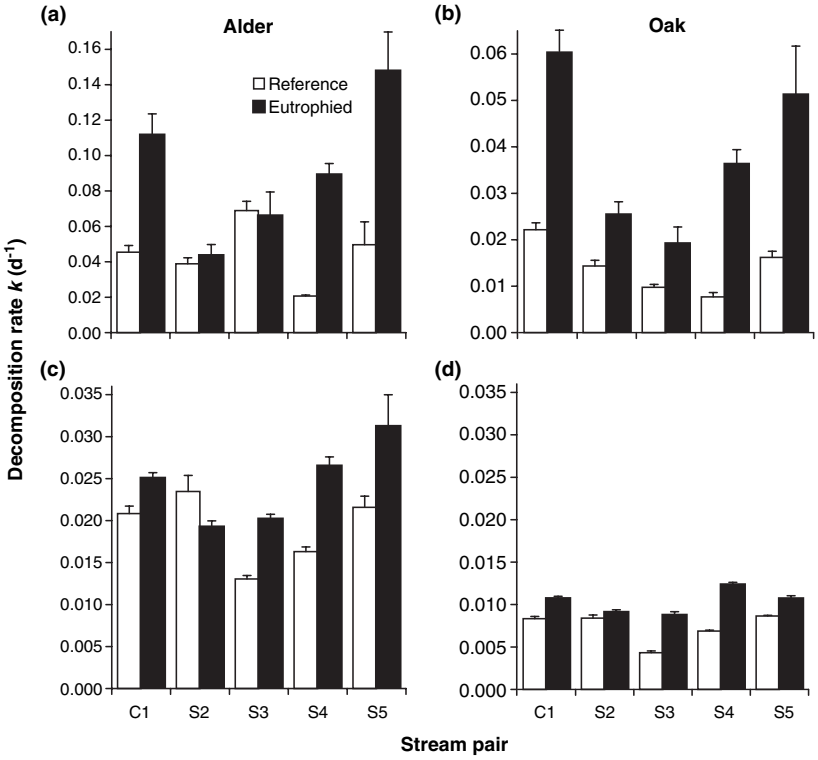

Fig. 1 Decomposition rates of alder and oak leaves in coarse (a, b) and fine (c, d) mesh bags in paired reference and eutrophied streams. Values are mean ± SE. See Table 1 for stream pair abbreviations.

coarse mesh bags in the eutrophied stream was different from all other treatments.

Nutrients in leaf litter

Nitrogen content of both alder and oak litter did not differ between the reference and the eutrophied stream (pair C1) throughout the decomposition (Fig. 2c,d; randomised block ANOVA, $P > 0.15$), but overall it was higher in fine than in coarse mesh bags (RB ANOVA, $P < 0.001$ and $P < 0.002$ for alder and oak respectively). In contrast, phosphorus content of both types of litter was significantly higher in the eutrophied stream (Fig. 2e,f; RB ANOVA, $P < 0.0002$). Oak leaves in fine mesh bags had higher phosphorus content than in coarse mesh bags (RB ANOVA, $P = 0.002$), while no difference for alder leaves was detected ($P = 0.85$).

Fungal parameters

Fungal biomass associated with both types of leaf litter in coarse mesh bags increased faster in the eutrophied stream than in the reference stream and also peaked at a higher level for oak (Fig. 3a,b), but the overall difference between streams was significant only for oak (RB ANOVA, $P = 0.0004$). Sporulation rate of aquatic hyphomycetes tended to peak earlier and at a higher level in the eutrophied stream (Fig. 3c,d; RB ANOVA, $P = 0.001$ and $P = 0.055$ for alder and oak respectively). No difference in fungal sporulation rate from alder leaves between mesh sizes was found (RB ANOVA, $P = 0.09$); samples from oak leaves in fine mesh bags were not analysed.

Macroinvertebrates

Total macroinvertebrate abundance associated with alder leaf litter in coarse mesh bags tended to be higher in eutrophied streams but did not differ significantly between stream types (paired *t*-test, $P = 0.138$; nested ANOVA (streams not paired), $F_{1,8} = 3.55$, $P = 0.088$) while taxa richness was slightly higher in eutrophied streams (paired *t*-test, $P = 0.037$; nested ANOVA, $F_{1,8} = 4.59$, $P = 0.060$) (Fig. 4a,c). Total invertebrate abundance and the number of taxa associated with oak leaves were both higher in eutrophied streams than in reference streams (paired *t*-test, $P = 0.030$ and $P = 0.012$; nested ANOVA, $F_{1,8} = 4.37$, $P = 0.070$ and $F_{1,8} = 12.07$, $P = 0.008$

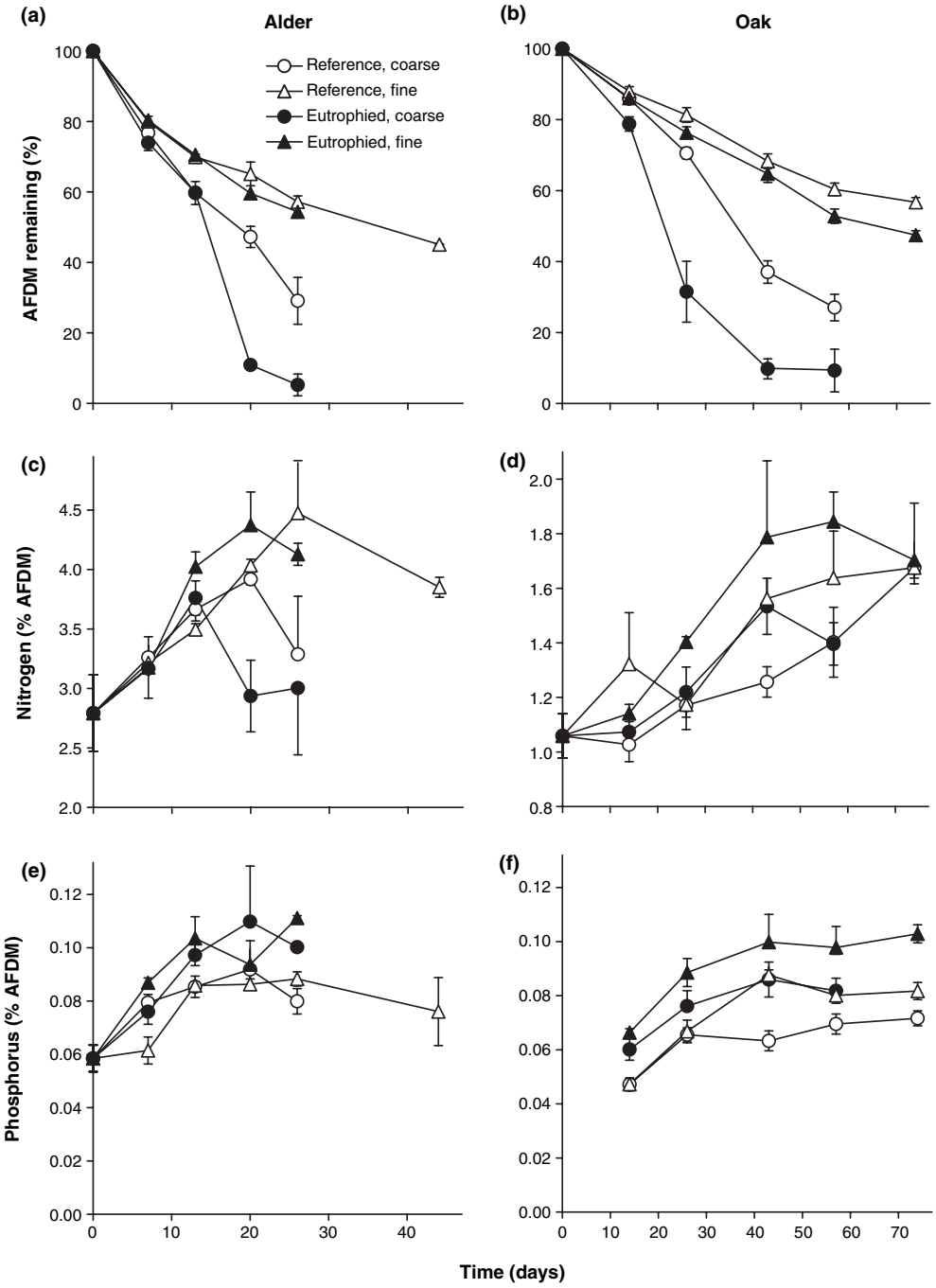

Fig. 2 Decomposition (a, b), nitrogen content (c, d) and phosphorus content (e, f) of alder and oak leaves in coarse and fine mesh bags in complete time series streams (stream pair C1, see Table 1). Values are mean ± SE.

respectively) (Fig. 4b,d). Shredder abundance and richness in litter bags was too low to warrant statistical analyses (across streams and leaf types abundance averaged 0.17–3.3 individuals g^{-1} AFDM and richness 0.9–1.2 taxa g^{-1} AFDM).

Comparison of selected macroinvertebrate parameters based on data from benthic macroinvertebrate communities from the 10 streams (Table 2) showed that % shredders, % shredder taxa, % EPT taxa and IBMWP index were significantly higher in reference

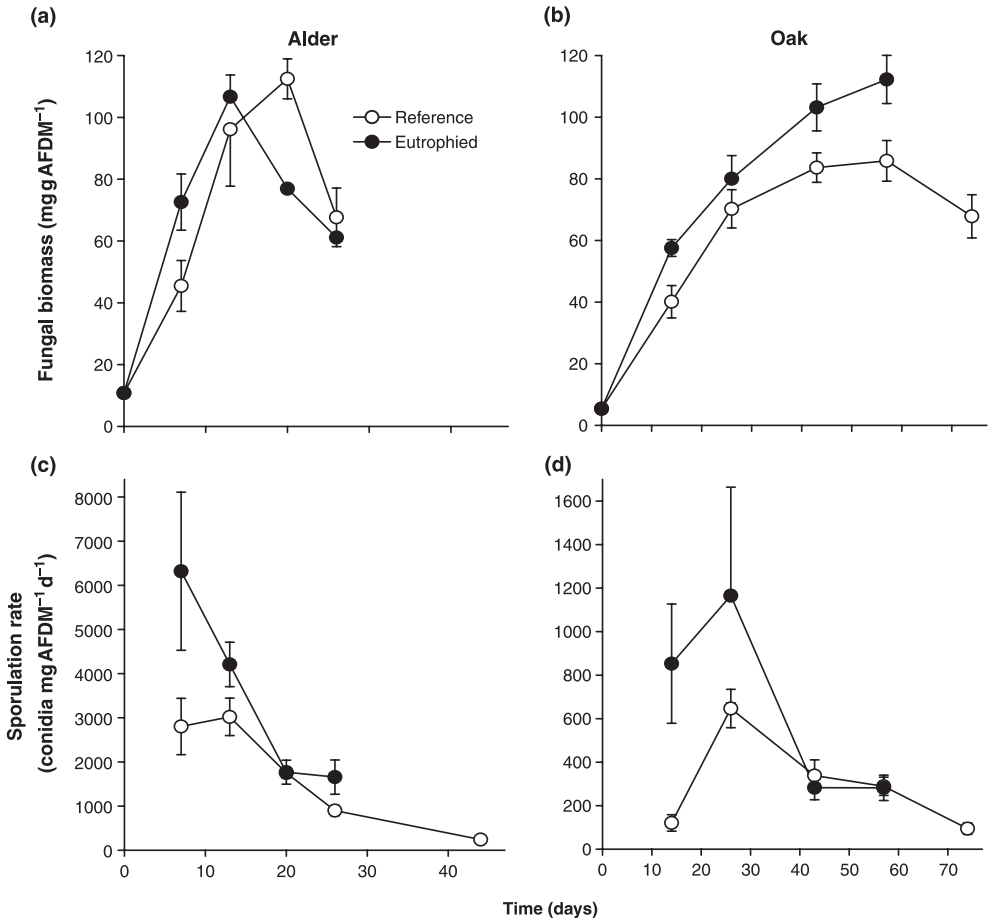

Fig. 3 Fungal biomass (a, b) associated with alder and oak litter in coarse mesh bags and sporulation rate of aquatic hyphomycetes (c, d) from leaf litter in complete time series streams (stream pair C1). Values are mean ± SE. Sporulation data from alder leaves in fine and coarse mesh bags were combined as no significant differences in sporulation from leaves enclosed in different mesh-size bags were found.

than in eutrophied streams (paired *t*-test, $P < 0.05$). However, the IBMWP biotic index response to reduced water quality was not sensitive enough to discriminate eutrophied streams as all of the streams in this study were classified as having 'very good' ecological conditions (the highest class for this index, Table 2).

Discussion

Moderate eutrophication is probably the most widely occurring type of stream pollution caused by agricultural practices (fertilisers, pastures, etc.), logging or atmospheric deposition. This type of pollution is often characterised only by increased nutrient concentrations in stream water, with low and unchanged levels of other pollutants, and our eutrophied streams fell into this category. In comparison with other studies in the Iberian peninsula, our eutrophied streams had similar nutrient concentrations to the 'reference' site used by Pascoal *et al.* (2005a) in Portugal or just a little higher nutrients than first order 'nutrient-poor' streams used by Molinero *et al.* (1996) in Spain. There are indications that dissolved inorganic nutrients have large effects on microbes and plant litter decomposition rates at relatively low concentrations (e.g. DIN < 300 μg L^{-1}, SRP < 50 μg L^{-1}, Gulis & Suberkropp, 2003b; Gulis *et al.*, 2004) and the effect appears to be asymptotic with low half saturation concentrations (Huryn *et al.*, 2002; Rosemond *et al.*, 2002; Ferreira *et al.*, 2006b). Consequently, the choice of truly oligotrophic reference streams is critical and it allowed us to detect differences caused by moderate eutrophication in decomposition rates, microbial and

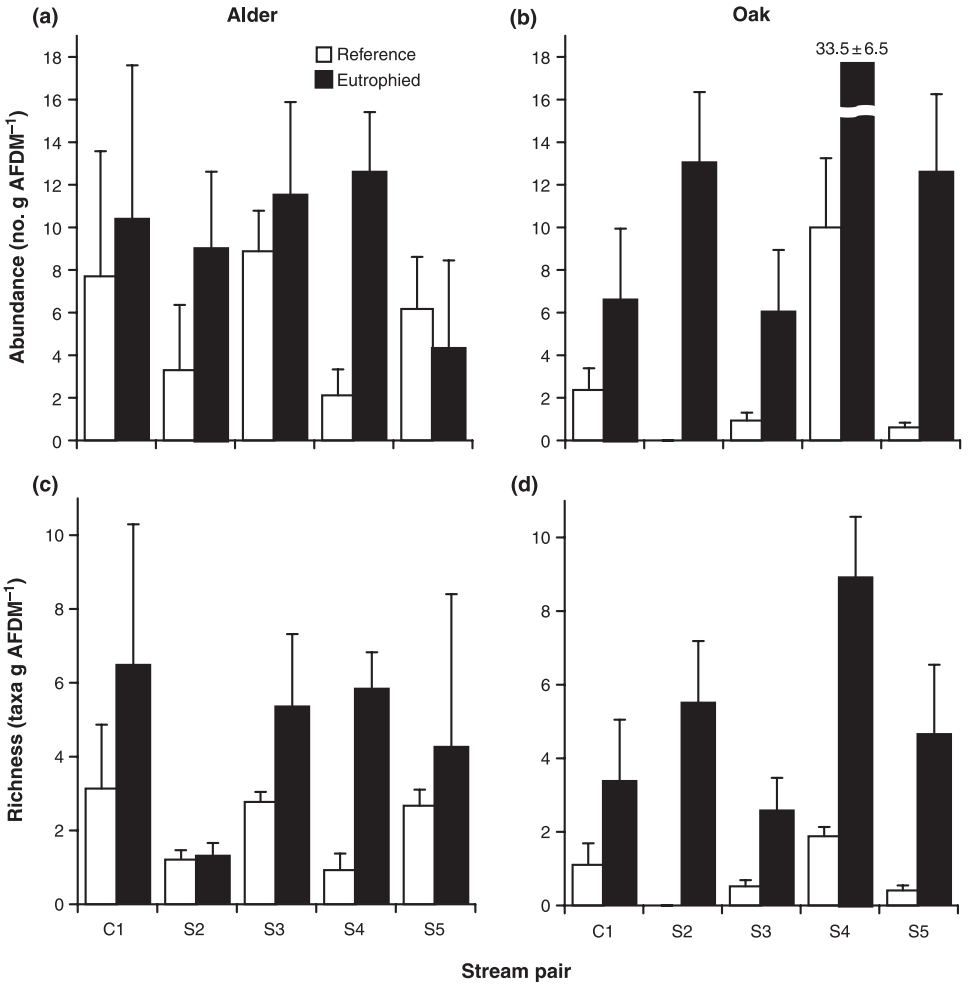

Fig. 4 Total invertebrate abundances (a, b) and taxa richness (c, d) associated with alder and oak leaf litter in coarse mesh bags in paired reference and eutrophied streams. Values are mean ± SE. See Table 1 for stream pair abbreviations. Invertebrates were collected on day 26 of the experiment from alder leaf bags and on day 57 from oak leaves bags in all streams except eutrophied streams of the pairs S4 and S5, from where they were collected on day 22 (alder) and day 42 (oak) because of faster decomposition rates and hence possibility of complete disappearance of leaf litter by the scheduled sampling dates.

invertebrate parameters associated with oak leaves in this study. Thus, the results of experimental whole stream nutrient additions (Elwood *et al.*, 1981; Gulis & Suberkropp, 2003b; Gulis *et al.*, 2004; Benstead *et al.*, 2005; Ferreira *et al.*, 2006b; Greenwood *et al.*, 2006) may be transferable to low or moderately eutrophied streams when the conditions described above are met.

Decomposition

On average, decomposition rates in eutrophied streams were 2.3 and 2.7 times higher for alder and oak leaves, respectively, than in reference streams in coarse mesh bags and 32% and 48% higher, respectively, in fine mesh bags (statistically significant for oak leaves only). The effect of eutrophication on decomposition of alder leaves varied greatly among stream pairs (Fig. 1) while for oak it was more uniform. This appears to be due to the much higher susceptibility of alder leaves to mechanical fragmentation and erratic shredder colonisation and feeding. Oak leaves have lower initial nutrient content than alder, thus fungi growing on oak leaves are more nutrient limited and, as microorganisms can use nutrients from both the substrate and overlying water (e.g. Suberkropp, 1998a), would respond readily to the extra nutrients in eutrophied streams. This resulted in slightly higher response of oak than alder litter

Table 2 Selected invertebrate parameters based on benthic samples from reference and eutrophied streams. Stream types were compared by paired *t*-test

Stream	Stream pair	% shredders	% shredder taxa	% EPT	% EPT taxa	IBMWP index
Reference streams						
Candal 1	C1	32.1	26.9	56.3	49.0	214
Sardeira	S2	45.7	22.5	87.0	59.7	150
Agueda	S3	44.9	26.4	73.3	57.3	134
Agadao	S4	18.5	30.6	40.0	47.4	138
Tojosa	S5	28.9	22.9	86.0	55.9	159
Eutrophied streams						
Casteloes	C1	21.9	17.7	59.6	44.2	179
Muceres	S2	7.8	15.1	45.7	46.3	119
Caramulo 1	S3	2.3	13.0	62.0	40.9	108
Caramulo 2	S4	12.2	16.4	33.7	39.1	128
Criz	S5	6.0	18.0	34.5	37.1	116
P (paired *t*-test)		0.029	0.005	0.113	0.009	0.006

EPT, Ephemeroptera + Plecoptera + Trichoptera; IBMWP, 'Iberian Biological Monitoring Working Party' index (Alba-Tercedor *et al.*, 2002; Jáimez-Cuéllar *et al.*, 2002). Scores above 100 indicate 'very good' (class 1 out of 5) ecological condition.

decomposition to eutrophication, which is consistent with earlier findings that both the decomposition of, and the microorganisms associated with substrates of high C : N and/or C : P ratios show greater responses to nutrient enrichment than those of high nutrient substrates (Stelzer *et al.*, 2003; Gulis *et al.*, 2004; Ferreira *et al.*, 2006b).

We found a significant relationship between SRP in stream water and leaf litter decomposition rate for the 10 streams in this study plus additional data points from companion studies (see Methods). Both linear and Michaelis–Menten saturation type models provided reasonable fits to the data (Fig. 5). The low half saturation constants K_m (i.e. SRP concentrations at which half rate of decomposition is achieved) for both alder and oak leaves are comparable with estimates by Rosemond *et al.* (2002). The Michaelis–Menten model provided a poor explanation of the relationship between DIN in stream water and decomposition rates (not shown). However, decomposition rates of

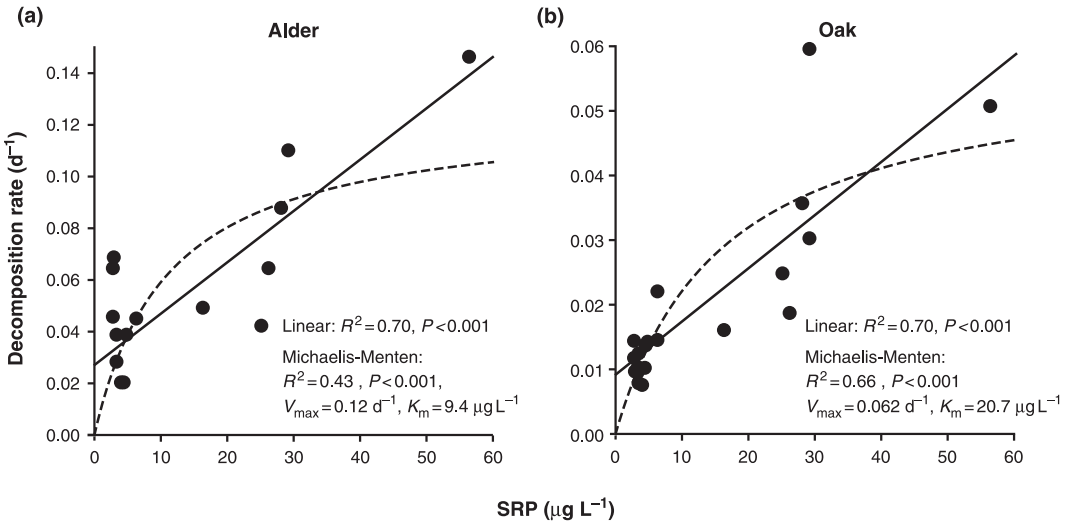

Fig. 5 Relationship between SRP concentration in stream water and decomposition rate of alder and oak leaves in coarse mesh bags in Portuguese streams (data from this and companion studies, see Methods; $n = 15$ for alder and $n = 18$ for oak). Reasonable fits to the data were given by both linear and Michaelis–Menten models. In this case the latter is: $V = (V_{max}[S])/(K_m + [S])$, where V_{max} is the maximum decomposition rate, K_m is the half saturation constant and $[S]$ is SRP concentration.

Table 3 Correlations (Pearson *r*) between nutrient concentrations in water and decomposition rates, fungal and invertebrate parameters associated with leaf litter in coarse mesh bags. Data points (N) from this and companion studies in Portuguese streams (see Methods) were used. Fungal parameters measured at early stages of decomposition (day 14) were used.

	Alder					Oak				
	DIN		SRP			DIN		SRP		
Parameter	*r*	*P*	*r*	*P*	N	*r*	*P*	*r*	*P*	N
Decomposition rate, coarse mesh bags	0.62	0.01	0.83	<0.001	15	0.57	0.03	0.86	<0.001	18
Decomposition rate, fine mesh bags	0.53	0.04	0.76	0.001	15	0.59	0.02	0.67	0.006	18
Fungal biomass						0.47	ns	0.40	ns	6
Sporulation rate						0.92	0.01	0.89	0.02	6
Invertebrate abundance	0.44	ns	0.15	ns	15	0.68	0.005	0.44	ns	17
Invertebrate taxa richness	0.59	0.02	0.41	ns	15	0.74	0.002	0.63	0.01	17

ns, not significant.

both leaf species in both types of mesh bags were positively linearly related to DIN and SRP (Table 3), which agrees well with findings from New Zealand streams impacted by agriculture that had ranges of nutrient concentrations similar to our streams (Niyogi *et al*., 2003) and also with the results from streams in Alabama (Suberkropp & Chauvet, 1995). Lecerf *et al.* (2006) studied decomposition in highly eutrophied hardwater streams in France, some of which had toxic levels of ammonium. They concluded that decomposition rate declined along the eutrophication gradient, which is opposite to our results from moderately eutrophied streams. It is plausible that the relationship between eutrophication and decomposition rate is unimodal on a large scale with decomposition being stimulated at low to moderate levels of eutrophication through microbial activity and depressed in highly eutrophied streams because of toxicity issues and presence of other pollutants with deleterious effects on biota.

Fungal parameters and nutrient content of leaf litter

Both fungal biomass accrual and sporulation rate of aquatic hyphomycetes associated with leaves peaked earlier and/or reached higher maxima in the eutrophied than in the reference stream in this study, which is not surprising as stimulation of leaf associated fungi by nutrients in laboratory microcosms (Suberkropp, 1998a; Sridhar & Bärlocher, 2000; Gulis & Suberkropp, 2003a) and in whole-stream nutrient addition experiments (Gulis & Suberkropp, 2003b; Stelzer *et al*., 2003; Gulis *et al*., 2004; Benstead *et al*., 2005; Ferreira *et al*., 2006b) is well documented. Generally, higher fungal biomass or activity were also reported from polluted streams that had elevated nutrient concentrations (Niyogi *et al*., 2003; Pascoal & Cássio, 2004) but in some cases the effect of nutrients could be masked by other factors such as low oxygen in organically polluted streams or pollutants with adverse effects on microorganisms (e.g. Raviraja *et al*., 1998; Pascoal *et al*., 2003; Pascoal, Cássio & Marvanová, 2005b). We found a positive correlation between sporulation rate of aquatic hyphomycetes associated with oak leaves at very early stages of decomposition and DIN and SRP in stream water (Table 3) based on data from this and a companion study in Portuguese streams (see Methods). Correlation between stream water NO_3-N but not PO_4-P and sporulation rate was reported before from streams in Alabama (Suberkropp & Chauvet, 1995). We did not find correlation between fungal biomass and dissolved nutrients while Niyogi *et al.* (2003) reported a positive relationship between fungal biomass associated with tussock grass and dissolved phosphorus.

Changes in nutrient content of decomposing plant litter are often attributed, at least in part, to microbial nutrient immobilisation from the water column (e.g. Chauvet, 1987; Hamilton *et al*., 2001; Gulis, Kuehn & Suberkropp, 2006). However, much higher availability of N in the eutrophied stream in this study did not result in its higher immobilisation while P content of leaf litter was higher in the eutrophied than in the reference stream. This suggests that microorganisms were probably P limited or co-limited by both N and P in the reference stream. Indeed, microbial N demand can be met at relatively low stream water N concentrations of *c.* 200 μg L^{-1} NO_3-N

according to some estimates (Huryn *et al.*, 2002; Ferreira *et al.*, 2006b), which is only two to three times the concentration observed in our reference stream. This also agrees with the better relationships between SRP than DIN and decomposition rates (Fig. 5, Table 3).

Macroinvertebrates

The tendency for higher macroinvertebrate abundance (this study) or biomass in litter bags in eutrophied streams or as a result of enrichment has been also reported by other authors (e.g. Robinson & Gessner, 2000; Niyogi *et al.*, 2003; Pascoal *et al.*, 2003; Greenwood *et al.*, 2006) and can be attributed to increased litter quality caused by elevated microbial activity. Both total invertebrate abundance and taxa richness associated with oak litter bags in Portuguese streams (data from this and companion studies, see Methods) were positively correlated with DIN and/or SRP in water (Table 3) while no negative correlations were found.

Benthic macroinvertebrate parameters based on community structure that are considered useful in stream assessment had an opposite trend in our streams. Percentage of shredders and shredder taxa, % EPT and % EPT taxa (Table 2 plus similar data from five additional reference streams from a companion study, see Methods) negatively correlated with both DIN and SRP in stream water (Pearson r ranged from -0.54 to -0.75, P ranged from 0.039 to <0.001), while no significant correlations were found for IBMWP. Lecerf *et al.* (2006) also reported a general decline in community-based benthic macroinvertebrate parameters along a eutrophication gradient. Many Plecoptera, Ephemeroptera and Trichoptera families are sensitive to organic pollution (and/or increases in inorganic nutrients that often coincide) and are assigned high scores in biotic indices (e.g. Tachet *et al.*, 2000; Jáimez-Cuéllar *et al.*, 2002). Previous studies in Portuguese streams reported use of macroinvertebrate metrics in classifying streams or reaches according to their overall pollution gradient (Pascoal *et al.*, 2003) or organic pollution (Pinto *et al.*, 2004). However, the IBMWP was evidently not sufficiently sensitive to detect the moderate eutrophication in our study.

Implications for stream assessment

Gessner & Chauvet (2002) made a strong case for using plant litter decomposition to assess functional stream integrity. They suggested using either (1) absolute values of decomposition rates k, (2) ratios of k in disturbed versus reference reaches or streams and (3) ratios of k in coarse mesh and k in fine mesh bags. The last two approaches may indeed add robustness and sensitivity to the tool (Gessner & Chauvet, 2002; Pascoal *et al.*, 2005a). For example, in our study, $k_{disturbed}/k_{reference}$ averaged 2.7 for oak leaves in coarse mesh bags. Decomposition rates in eutrophied streams increased to a greater extent in coarse than in fine mesh bags that resulted in statistically significant shift in the k_{coarse}/k_{fine} ratio for oak leaves (Table 4). The twofold increase in the ratio in eutrophied streams may be explained by increased microbial activity and leaf conditioning that made leaves more susceptible to secondary agents of decomposition in coarse mesh bags such as macroinvertebrate feeding and physical fragmentation and

Table 4 Ratios of leaf litter decomposition rates in coarse mesh bags to decomposition rates in fine mesh bags (k_{coarse}/k_{fine}) for both leaf types in reference and eutrophied streams

	Alder		Oak	
Stream pair	Reference	Eutrophied	Reference	Eutrophied
C1	2.18	4.45	2.66	5.20
S2	1.66	2.23	1.71	2.82
S3	5.28	3.25	2.25	2.20
S4	1.27	3.33	1.12	2.96
S5	2.30	4.70	1.87	4.85
Mean k_{coarse}/k_{fine} ratio	2.54	3.59	1.92	3.72
P (paired t-test)	0.277		0.039	
P (nested ANOVA)*	0.099		0.007	

*Nested ANOVA was done on ash-free dry mass remaining data for each fine/coarse mesh bag pair, streams were not paired.

may have changed the relative importance of the breakdown agents. Clearly, a sizeable database regarding this ratio is needed to ensure comparison of the stream in question with reference streams of the same type (order, geology, substrate, etc.). We feel that the tentative values suggested by Gessner & Chauvet (2002) may need upward revision as is also evident from the report by Lecerf *et al.* (2006). We also suggest that slowly decomposing, tough, low-nutrient substrates such as oak leaves, or even wood veneers, are better suited for decomposition assays because they combine the greater response of microorganisms to nutrients in water, and hence higher sensitivity, with lower overall variability because they are less susceptible to mechanical fragmentation and atypical results due to flood events.

While average overall differences in decomposition rates in coarse mesh bags between reference and eutrophied streams in this study were as much as 2.3- to 2.7-fold, all of the streams were actually classified as having 'very good' ecological conditions (highest class) according to the IBMWP benthic macroinvertebrate index. This indicates that plant litter decomposition responds to even low levels of eutrophication and therefore could be a useful functional measure to complement assessment programs based on structural parameters.

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