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GLOBAL CHANGE ECOLOGY – ORIGINAL RESEARCH

Nutrient enrichment in water more than in leaves affects aquatic microbial litter processing

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Abstract Nutrient enrichment of soils and water will intensify in the future and has the potential to alter fundamental ecosystem processes, such as litter decomposition. We tested the direct (via water nutrient enrichment) and indirect (via changes in leaf chemistry) effects of nutrient enrichment on microbial activity and decomposability of *Quercus robur* L. (oak) leaves in laboratory microcosms simulating streams. Senescent leaves of oak trees grown without and with fertilization were incubated under ambient and elevated water nutrient [nitrogen (N) and phosphorus (P)] concentrations for 60 days. Soil fertilization led to an increase in leaf (3.4×) and leaf litter (2.3×) N concentration. Increased water-dissolved nutrients concentrations stimulated microbial activity (N uptake, microbial respiration, fungal biomass buildup and conidia production by aquatic hyphomycetes) that translated into accelerated litter decomposition (2.1× for unfertilized and 1.6× for fertilized trees). Leaves from fertilized trees had higher microbial activity and decomposition rates than leaves from unfertilized trees only at low dissolved nutrient availability. When both litter and water nutrients concentration increased, microbial activity and leaf decomposition were stimulated, but the effects were additive and direct effects from increased dissolved nutrient availability were stronger than those mediated by increases in litter N concentration (indirect effects). Our results suggest that increases in water nutrient availability (within the range used in this study) may exert a stronger control on microbial activity and litter decomposition than litter nutrient enrichment.

Keywords Eutrophication · Fertilization · Litter · Aquatic hyphomycetes · Decomposition · Streams

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Introduction

Human activity has become the primary driver of global environmental change (Rockström et al. 2009). One of the most pervasive human impacts on both terrestrial and aquatic systems is the increase in nutrient availability (Rockström et al. 2009), caused mainly by the use of fertilizers (Bowman et al. 2008; Galloway et al. 2008). Additionally, atmospheric nitrogen (N) deposition is a significant contributor to ecosystems nutrient enrichment, particularly in western Europe, eastern USA and China (Holland et al. 2005; Zhang et al. 2012; Liu et al. 2013; Meunier et al. 2016). As agriculture intensifies to feed a growing human population (Galloway and Cowling 2002) and atmospheric N deposition rises due to increases in the combustion of fossil fuels and intensification in agriculture and cattle production (Liu et al. 2013), nutrient enrichment of soils and water will also intensify in the future (MEA 2005). Increased nutrient availability has the potential to alter fundamental ecosystem processes and thus affects ecosystem services (Woodward et al. 2012).

Plant litter decomposition is an essential ecosystem process in small forest streams, where shade by the riparian vegetation limits instream primary production, and terrestrially derived leaf litter constitutes a primary source of carbon and energy for aquatic food webs (Vannote et al. 1980; Wallace et al. 1997). The decomposition of this leaf litter is largely mediated by aquatic microbes, especially aquatic hyphomycetes (Hieber and Gessner 2002; Gulis and Suberkropp 2003a; Pascoal and Cássio 2004). Microbes macerate the litter by the activity of their external enzymes, mineralize organic carbon through respiration, convert it into mycelium and reproductive structures (conidia) and promote the release of fine particulate organic matter, which leads to leaf litter mass loss (Hieber and Gessner 2002; Gulis and Suberkropp 2003a, b; Cornut et al. 2010).

Microbial decomposers can use nutrients from both the organic substrate and the water, but dissolved nutrients are already in their inorganic form and are easier to uptake (Suberkropp and Chauvet 1995; Suberkropp 1998; Gulis and Suberkropp 2003a, b). Increases in nutrient availability resulting from human activity can thus affect microbial activity and litter decomposition directly by increasing inorganic nutrients concentration in water. Indeed, stream nutrient enrichment stimulates microbial metabolism, growth and reproduction (Suberkropp and Chauvet 1995; Gulis and Suberkropp 2003b; Gulis et al. 2004, 2006; Ferreira et al. 2006) and consequently litter decomposition (Ferreira et al. 2015).

Human-induced increases in nutrient availability can also affect microbes and litter decomposition indirectly by changing leaf litter properties, including leaf nutrient concentration since leaf traits are related to local environmental conditions including soil nutrient availability (Reich and Oleksyn 2004). Plants from nutrient-rich soils tend to have higher leaf N and phosphorus (P) concentration and lower lignin concentration and specific leaf area than plants from nutrient-poor soils (Lecerf and Chauvet 2008; Liu et al. 2010; Graça and Poquet 2014). Traits such as toughness, lignin and nutrient concentration (mainly N and P) and plant chemical defenses are predictors of leaf litter decomposition rates. Soft litter, with high nutrient concentration and low lignin concentration, generally supports higher microbial activity and decomposes faster than more recalcitrant litter (Gessner and Chauvet 1994; Lecerf and Chauvet 2008; Graça and Cressa 2010; Ferreira et al. 2012). This trend is also found within given species as a result of high intraspecific variability in leaf traits (LeRoy et al. 2007; Lecerf and Chauvet 2008; Graça and Poquet 2014). Several studies have addressed the simultaneous effects of water nutrient enrichment and changes in leaf characteristics on microbial litter decomposition by using different leaf species. These studies generally show a stronger stimulation of microbial activity and litter decomposition with water nutrient enrichment for litter with low than high nutrient concentration due to a stronger nutrient limitation of microbes in the nutrient-poor substrates (Gulis and Suberkropp 2003a, b; Ferreira et al. 2006; Gulis et al. 2006; Ardón et al. 2009; but see Fernandes et al. 2014). Changes in dissolved nutrient availability and in litter quality also generally affect aquatic hyphomycete community structure since different species have different enzymatic capabilities and nutrient requirements (Chandrashekar and Kaveriappa 1988; Bisht 2013; Grimmett et al. 2013; Danger et al. 2016). However, no previous study investigated how litter decomposition is simultaneously affected by water nutrient enrichment and litter nutrient enrichment.

Here we tested the direct (via water nutrient enrichment) and indirect (via changes in leaf chemistry caused by fertilization) effects of nutrient enrichment on microbial activity (overall microbial metabolism, fungal somatic growth and aquatic hyphomycete reproduction) and leaf decomposition in laboratory microcosms simulating streams. Senescent leaves of *Quercus robur* L. (oak) trees grown without and with fertilization, and thus different in chemical composition, were incubated under ambient and elevated water nutrient concentration. Since the nutrient concentration in the soil potentially influences the chemical composition of the leaves, we expected that increases in soil fertilization would lead to the production of leaves with higher nutrient concentration and lower concentrations of structural and secondary compounds when compared with leaves from trees in unfertilized soils (Lecerf and Chauvet 2008; Liu et al. 2010; Graça and Poquet 2014). We expected that less recalcitrant leaves (e.g. higher nutrient concentration, lower concentration of structural compounds) would support higher microbial activity and decompose faster than more recalcitrant leaves (Gessner and Chauvet 1994; Lecerf and Chauvet 2008; Ferreira et al. 2012). Nutrient enrichment of stream waters generally stimulates microbial activity and litter decomposition, and thus we expected that increases in water nutrient concentration would promote microbial activity and litter decomposition (Suberkropp and Chauvet 1995; Gulis and Suberkropp 2003a, b; Ferreira et al. 2006, 2015). The relative importance of the direct and indirect effects of nutrient enrichment on microbial activity and litter decomposition is harder to anticipate, but we expected a synergistic interaction since individual increases in nutrient concentration in leaves and in water generally stimulate microbial activity, i.e. affect microbial activity in the same direction. Changes in leaf chemical composition and water nutrient availability were also predicted to affect aquatic hyphomycete community structure (Gulis and Suberkropp 2003a, b; Ferreira et al. 2006).

Materials and methods

Trees and leaf litter

To test the degree to which leaf litter traits responded to fertilization, ten *Q. robur* trees of 1.7–2.0 m height (about 2-year old) were planted individually in 40-L buckets containing 50% sand and 50% ground pine bark as a substrate. Trees were kept in the open air in a garden from April to November 2015, at Semide, central Portugal (mean annual air temperature and precipitation: 15.5 °C and 999 mm, respectively). Trees were randomly allocated into two treatments, without (NoFert) and with (Fert) fertilization ($n = 5$ for each treatment). At monthly intervals (May to October 2015), ~17.7 g of fertilizer (Compo Blaukorn®, Münster, Germany) was added to the five trees in the Fert treatment; this corresponds to monthly additions of 2.625 g of N, 0.458 g P, 1.743 g K, 0.210 g Mg, 1.575 g Na, 3.5 mg B and 1.8 mg Zn. Trees were daily watered (~ 1 L). In fall 2015 (October – November), senescent leaves were collected individually (per tree), air dried at room temperature and stored in paper bags in the dark until used.

Leaf litter conditioning

Leaf discs (12-mm Ø) were cut with a cork borer from moistened leaves, avoiding primary and secondary veins, oven-dried (40 °C, 48 h) and weighed in groups of 20 discs (±0.01 mg). Each group of discs was enclosed into 500-µm mesh bags (5 × 7 cm) and bags were incubated in a 20-L tank containing unfiltered stream water and ~8 L of a diverse litter mixture composed of leaves at different stages of degradation for microbial colonization. Both water and litter were collected from a local oligotrophic stream (Ribeira do Candal, Lousã Mountain, central Portugal; 40º4′44′′N, 8º12′19′′W, 620 m asl), flowing through a broadleaf deciduous forest dominated by chestnut (*Castanea sativa* Mill.) and oak trees (see Ferreira et al. (2012) for environmental details). The water in the tank was renewed every two days to prevent the accumulation of polyphenols leached from the litter, which could inhibit microbial colonization of leaf discs. The tank was kept at 18 °C, under 12 h light:12 h dark regime and with strong aeration for one week to ensure inoculation of leaf discs by near-natural microbial decomposer assemblages.

After one-week conditioning, each group of discs was used in a microcosm experiment (see below). Discs dry mass (DM) at day 0 was estimated by multiplying DM before conditioning by a conversion factor derived from an additional group of discs from each tree that was conditioned for one week. These discs were dried at 40 °C for 48 h and weighed (±0.01 mg) for determination of DM after conditioning, and the before-to-after DM conversion factor was calculated as the ratio between DM after conditioning to DM before conditioning.

Leaf chemical and physical properties

Leaf toughness was determined for ten discs from each tree with a penetrometer after discs had been soaked in distilled water for 1 h, with results expressed as the mass (g) required to force a blunt iron rod (1.55 mm Ø) through the disc (Graça et al. 2005). Specific leaf area (SLA) was determined for the same leaf discs after drying at 40 °C for 48 h and expressed as $mm^2\ mg^{-1}$. Green leaves (collected from trees by the end of September), senescent leaves and leaf discs (before and after conditioning) were ground to powder (1 mm screen; Retsch MM 400, Haan, Germany) and analysed for carbon (C) and N concentrations (IRMS Thermo Delta V advantage with a Flash EA-1112 series; Thermo Fisher Scientific Inc., Waltham, MA, USA; $n = 3$ per tree). Lignin (Goering and Van Soest 1970), total polyphenols (Graça et al. 2005) and P concentrations (APHA 1995) were also determined for senescent leaves ($n = 3$ per tree). Results of chemical analyses were expressed as percentage of dry mass (% DM).

Microcosms

Laboratory microcosms consisted of 100-mL Erlenmeyer flasks supplied with 40 mL of either a low (LowNP, $n = 30$) or a high (HighNP, $n = 30$) nutrient solution, renewed three times a week. The LowNP solution contained per litre of distilled water 0.055 mg K_2HPO_4, 1 mg KNO_3, 75.5 mg $CaCl_2$, 10 mg $MgSO_4.7H_2O$ and 0.5 g morpholino-propane sulfonic acid (corresponding to 0.01 mg P L^{-1} and 0.14 mg N L^{-1}, which is within the values found in oligotrophic streams in central Portugal; Gulis et al. 2006), while the HighNP solution was amended with 5.5 mg K_2HPO_4 L^{-1} and 100 mg KNO_3 L^{-1} (corresponding to 0.98 mg P L^{-1} and 13.9 mg N L^{-1}) (adapted from Dang et al. (2005)). Microcosms were displayed on an orbital shaker (100–117 rotations min^{-1}; GLF 3017, Burgwedel, Germany) at 18 °C and under a 12 h light:12 h dark regime.

Half of the microcosms in each nutrient level ($n = 15$) received a group of conditioned leaf discs (see above) from either NoFert or Fert trees so that there were four treatments in a complete factorial design: (1) leaves from unfertilized trees incubated in low nutrient solution—NoFert, LowNP, (2) leaves from unfertilized trees incubated in high nutrient solution—NoFert, HighNP, (3) leaves from fertilized trees incubated in low nutrient solution—Fert, LowNP and (4)

leaves from fertilized trees incubated in high nutrient solution—Fert, HighNP ($n = 15$ for each; Online resource 1).

After 15, 35 and 60 days of incubation, five microcosms per treatment (i.e. one per tree) were sampled for the determination of leaf mass, leaf N and C concentrations, leaf toughness, microbial respiration rate, fungal biomass and conidia production by aquatic hyphomycetes (see below).

Conidia production by aquatic hyphomycetes

When microcosms were sampled, the conidial suspensions were saved into 50-mL Falcon tubes, preserved with 2 mL of 37% formalin and stored in the dark until processed. Before conidial counting and identification, 150 µL of polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100, 0.5%) was added to the suspensions and mixed with a magnetic stirring bar to ensure a uniform distribution of conidia, and an aliquot of the suspension was filtered through cellulose nitrate filters (25 mm Ø, 5 µm pore size; Sartorius Stedim Biotech GmbH, Göttingen, Germany). Filters were stained with 0.05% trypan blue in 60% lactic acid, and spores were identified and counted under a microscope at 200× magnification (Wild, Heerbrugg, Switzerland) according to Graça et al. (2005). To standardize the species richness by sample size, ~200 conidia were counted per sample. Sporulation rates, a measure of reproductive activity, were expressed as no. conidia released mg^{-1} leaf DM day^{-1} and aquatic hyphomycete species richness as no. species $sample^{-1}$.

Microbial respiration rate

Five discs were removed from each sampled microcosm to determine overall microbial metabolism as microbial oxygen consumption rates using a closed six-channel dissolved oxygen measuring system (Strathkelvin 929 System, North Lanarkshire, UK). The oxygen electrodes were calibrated against a saturated solution of sodium sulphite in 0.01 M sodium borate (0% O_2) and a 100% O_2-saturated low or high nutrient solution at 18 °C. Leaf discs were incubated in 3-mL chambers filled with the corresponding 100% O_2-saturated nutrient solution kept at 18 °C by a water bath. Additional chambers without leaf discs were used as controls. After a ~20–60 min trial, leaf discs were saved, oven-dried (40 °C, 48 h) and weighed (±0.01 mg) for determination of DM. Oxygen consumption rates were determined by the difference in the O_2 concentration in the sample and the control over a 15-min interval during which O_2 consumption over time was linear (between 5 and 20 min incubation). Results were expressed as mg O_2 g^{-1} leaf DM h^{-1}.

Fungal biomass

Five additional leaf discs from each microcosm were frozen at −20 °C, lyophilized overnight, weighed (±0.01 mg) to determine DM and used for ergosterol extraction as a surrogate for mycelial biomass (Gessner and Chauvet 1993; Graça et al. 2005). Lipid extraction and saponification were carried out in 10 mL KOH/methanol (8 g L^{-1}) at 80 °C for 30 min. The extract was purified by solid-phase extraction (Waters Sep-Pak © Vac RC tC18 cartridges; Waters Corp., Milford, MA, USA) and quantified with high-performance liquid chromatography (HPLC; Dionex DX-120, Sunnyvale, CA, USA) by measuring absorbance at 282 nm. The HPLC system was equipped with the Thermo Scientific Syncronis C18 column (250 × 4 mm, 5 µm particle size; Thermo, Waltham, MA, USA) and the Thermo Universal Unigard holder 4/4.6 mm ID3 + Syncronis C18 (10 × 4 mm, 5 µm particle size) drop in guard pre-column (Thermo), maintained at 33 °C. The mobile phase was 100% methanol and the flow rate was set to 1.4 mL min^{-1}. Fungal biomass was estimated from ergosterol assuming 5.5 µg ergosterol mg^{-1} fungal DM and the results were expressed as mg fungal DM g^{-1} leaf DM.

Leaf litter toughness, decomposition and nutrient concentration

The remaining ten leaf discs from each microcosm were used for toughness determinations, as a surrogate for enzymatic maceration of leaf litter, as described above. Leaf toughness was expressed as percentage of loss relative to initial (day 0) values. Further, these discs were dried at 40 °C for 48 h and weighed (±0.01 mg) for determination of DM. The DM of all three sets of discs from each microcosm was added up for determination of percentage of DM remaining. The ten leaf discs used only for DM determination were ground to powder and analysed for C and N concentrations, as described above. Nitrogen concentration and C:N ratio were expressed as percentage of change relative to initial (day 0) values.

Data analysis

Leaf toughness, SLA, polyphenols, lignin, C, N and P concentrations and C:N ratio were compared between fertilization levels (NoFert vs. Fert) for senescent leaves by *t* tests. Nitrogen and C concentrations and the C:N ratio were compared between fertilization levels (NoFert vs. Fert) and between (1) green and senescent leaves to assess changes in nutrient concentrations due to nutrient reabsorption during senescence, (2) senescent leaves and leaf discs before conditioning to assess if leaf discs quality would be representative of leaf quality and (3) leaf discs before and after conditioning to assess changes in nutrient concentration due to leaching

and microbial conditioning, by two-way analysis of variance (ANOVA; fertilization and leaf state as categorical factors).

The fraction of DM remaining (ln-transformed) over time was compared among treatments by analysis of covariance (ANCOVA; fertilization and water nutrients as categorical factors, time as covariate), which compared the parallelism of the regression lines (ln fraction of DM remaining versus time), followed by Fisher's test for multiple comparisons. Exponential decomposition rates on a per day basis (k, day^{-1}) were estimated for each treatment by linear regression of ln-transformed fraction of DM remaining over time (negative exponential model with fixed intercept). The observed (obs) and expected (exp) decomposition rates in a future scenario of nutrient enrichment (Fert, HighNP) were compared to assess the type of interaction between fertilization and water nutrients: obs ~ exp indicates additive effects, obs > exp indicates synergistic effects and obs < exp indicates antagonistic effects. The expected decomposition rate in a Fert, HighNP scenario was estimated from the stimulation of leaf decomposition observed in a present non-impacted scenario (NoFert, LowNP) by fertilization alone (Fert, LowNP) plus the stimulation by increases in water nutrients alone (NoFert, HighNP), assuming no interaction between factors.

Percentage of change in N concentration ($\log(x + 66.2)$), C:N ratio ($\log(x + 76.8)$; (constants added to get only positive values) and toughness (acosin), microbial respiration rate ($\log(x + 1)$), fungal biomass, sporulation rate ($\text{sqrt}(\log(x + 1))$) and species richness of aquatic hyphomycetes was compared among treatments by two-way repeated measures ANOVAs (fertilization and water nutrients as categorical factors) followed by Tukey's test, or Fisher's test when Tukey's test was not able to identify differences for significant factors in ANOVA. Ordination of aquatic hyphomycete communities was done by non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarity matrix of conidial production data ($\log(x + 1)$). Analysis of similarity (ANOSIM) was used to compare aquatic hyphomycete communities among treatments (Primer 6 v6.1.11 and Permanova + v1.0.1; Primer-E Ltd, Plymouth, UK; Clarke and Gorley 2001).

Data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Bartlett's test) and transformed when necessary. Analyses were performed using R 3.1.1 (R Core Team 2014), except when indicated otherwise.

Results

Leaf chemical and physical properties

Senescent leaves from unfertilized and fertilized trees did not significantly differ in toughness, SLA, polyphenols, lignin, C and P concentrations (Table 1). However, fertilization resulted in a significantly higher N concentration in green leaves (3.4×), senescent leaves (2.3×), pre-conditioned leaf discs (2.2×) and conditioned leaf discs (1.7×) when compared to the unfertilized treatment (Table 2 and Online resource 2). Consequently, the C:N ratio was significantly higher in the unfertilized than fertilized treatment for green leaves (3.6×), senescent leaves (2.3×), leaf discs before conditioning (2.3×) and leaf discs after conditioning (1.8×) (Table 2 and Online resource 2).

Nitrogen concentration in leaves from fertilized trees decreased by 48% with senescence, while no significant reduction occurred in leaves from unfertilized trees; senescence also resulted in a small but significant reduction in C concentration in both fertilized and unfertilized treatments (Table 2 and Online resource 2). There were no significant differences in N and C concentrations and in the C:N ratio between senescent leaves and leaf discs before conditioning and between leaf discs before and after conditioning (Table 2 and Online resource 2).

Leaf decomposition

Leaf discs lost 17–31% of their initial mass during the 60 days incubation (Fig. 1a), which translated into decomposition rates (k) of 0.0032–0.0073 day^{-1}. Decomposition rates were significantly faster at high than at low water nutrient concentration (ANCOVA, $p < 0.001$), while there was no significant effect of soil fertilization ($p = 0.144$) or interaction between soil fertilization and water nutrients ($p = 0.700$) (Table 3), despite the effect of the increase in water nutrient concentration on decomposition rates being stronger for leaves from NoFert than Fert trees (2.1× and 1.6×, respectively).

The decomposition rate of leaves from fertilized trees incubated at high nutrient concentration (Fert, HighNP) was significantly faster (2.3×) than that of leaves from unfertilized trees incubated at low nutrient

Table 1 Physical and chemical characteristics (mean ± SE, $n = 5$) of senescent leaves originating from unfertilized (NoFert) and fertilized (Fert) oak trees (t tests with p values)

Leaf characteristics	NoFert	Fert	p
Toughness (g)	162.6 ± 15.9	176.9 ± 17.3	0.280
SLA (mm^2 mg^{-1})	13.8 ± 1.4	12.4 ± 1.1	0.059
Polyphenolics (% DM)	12.7 ± 1.9	10.6 ± 1.6	0.222
Lignin (% DM)	34.3 ± 1.5	33.9 ± 1.4	0.431
Carbon (% DM)	47.2 ± 0.5	47.6 ± 0.3	0.260
Nitrogen (% DM)	0.60 ± 0.06	1.36 ± 0.06	<0.001
Phosphorus (% DM)	0.14 ± 0.05	0.11 ± 0.01	0.276
Carbon:nitrogen (molar)	95.8 ± 8.2	41.1 ± 1.8	<0.001

SLA specific leaf area, *DM* dry mass

Table 2 Nitrogen and carbon concentrations and carbon:nitrogen ratio (C:N) (mean ± SE, $n = 5$) of oak leaves in different stages originating from unfertilized (NoFert) and fertilized (Fert) trees

Leaf state	Fertilization	Nitrogen (% DM)	Carbon (% DM)	C:N (molar)
Green leaves	NoFert	0.76 ± 0.16	50.0 ± 0.6	64.3 ± 9.5
	Fert	2.60 ± 0.43	50.4 ± 0.6	17.9 ± 2.6
Senescent leaves	NoFert	0.60 ± 0.06	47.2 ± 0.5	70.4 ± 6.0
	Fert	1.36 ± 0.06	47.6 ± 0.3	30.2 ± 1.3
Leaf discs (pre-conditioned)	NoFert	0.62 ± 0.05	47.7 ± 0.7	71.0 ± 6.5
	Fert	1.35 ± 0.06	47.5 ± 0.4	30.4 ± 1.2
Leaf discs (post-conditioned)	NoFert	0.82 ± 0.14	47.6 ± 0.6	54.2 ± 6.9
	Fert	1.40 ± 0.06	47.7 ± 0.3	29.5 ± 1.5

DM dry mass

concentration (NoFert, LowNP) (Fisher's test, $p < 0.001$). The observed decomposition rate of leaves from fertilized trees incubated at high nutrient concentration (Fert, HighNP, 0.0073 day^{-1}) was 8% lower than expected (0.0080 day^{-1}) based on the individual effects of soil fertilization and increase in water nutrient concentration.

Leaf N concentration and C:N ratio

Nitrogen concentration in leaves incubated in water with high nutrients increased during decomposition up to days 15–35, while no major change was observed in the other treatments (Fig. 1b). There was an effect of soil fertilization and water nutrients on the magnitude of change in N concentration (two-way repeated measures ANOVA, $p = 0.002$ and $p < 0.001$; Fig. 1b and Online resource 3). No significant interaction between soil fertilization and water nutrients was detected (two-way repeated measures ANOVA, $p = 0.863$; respectively; Online resource 3) but the magnitude of change in N was significantly higher for leaves from unfertilized trees at high water nutrients than for any other treatment (Tukey's test, $p < 0.044$) (Fig. 1c).

The C:N ratio dynamics was opposite to that of N. The magnitude of change in the C:N ratio was significantly affected by fertilization (two-way repeated measures ANOVA, $p < 0.001$), water nutrient concentration ($p < 0.001$), the interaction between both factors ($p = 0.017$) and the interaction between fertilization and time ($p = 0.014$) (Online resource 3). The magnitude of change was significantly higher for leaves from unfertilized trees at high water nutrients than for leaves from any other treatment (Tukey's test, $p = 0.010$) (Fig. 1c).

Leaf toughness

Leaf toughness decreased sharply during the first two weeks of incubation after which toughness loss was less abrupt (two-way repeated measures ANOVA, $p = 0.020$) and it reached 45–18% of the initial value by day 60 (Fig. 2a). Leaf toughness loss was not significantly affected by tree fertilization (two-way repeated measures ANOVA, $p = 0.490$), while the effect of water nutrients depended on fertilization ($p = 0.039$) (Online resource 4). For leaves from unfertilized trees, leaf toughness loss was significantly higher at high than low water nutrients (Tukey's test, $p < 0.001$; Fig. 2a). Leaves from fertilized trees incubated at high water nutrient concentration had significantly higher toughness loss than leaves from unfertilized trees incubated at low water nutrient concentration (Tukey's test, $p = 0.002$).

Microbial respiration

Microbial respiration rates varied between 0.23 and 0.73 mg O_2 g^{-1} DM h^{-1} (Fig. 2b). Respiration rates were already high by day 15 for leaves incubated at high water nutrients and decreased thereafter, while for leaves incubated at low water nutrient concentration respiration rates were relatively stable over the incubation period (Fig. 2b). Respiration rates were significantly affected by water nutrients, with higher values at high than low nutrient concentration (two-way repeated measures ANOVA, $p < 0.001$), while no significant effect was found for fertilization ($p = 0.058$) or for the interaction between factors ($p = 0.635$) (Online resource 4). There was an effect of time and of the interaction between water nutrient concentration and time (two-way repeated measures ANOVA, $p = 0.013$ and $p = 0.009$, respectively; Online resource 4). Microbial respiration rates by day 15 were significantly higher for leaves incubated at high than low water nutrients (Fisher's test, $p < 0.001$; Fig. 2b). At the end of the experiment (day 60), respiration rates were significantly higher for leaves from fertilized trees incubated at high water nutrients than for other treatments (Fisher's test, $p = 0.008$–0.051). Leaves from fertilized trees incubated at high water nutrients had significantly higher respiration rates than leaves from unfertilized trees incubated at low water nutrients (Fisher's test, $p < 0.001$–0.051, depending on the date).

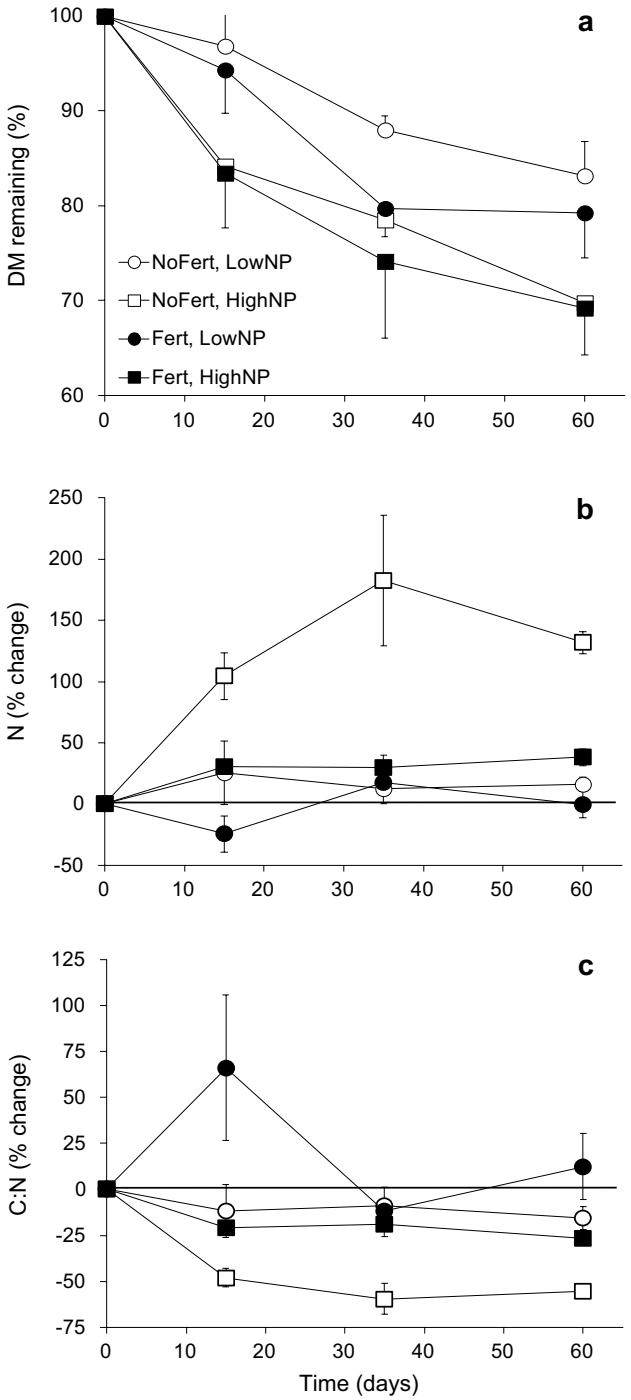

Fig. 1 Dry mass (DM) remaining (**a**), change in nitrogen (N) concentration (**b**) and change in carbon: nitrogen ratio (C:N) from initial values (**c**) of leaves originating from unfertilized (NoFert) and fertilized (Fert) trees and incubated in laboratory microcosms under low (LowNP, $n = 30$) and high (HighNP, $n = 30$) nutrient concentrations for 15, 35 and 60 days. Values are mean ± SE

Fungal biomass

Fungal biomass varied between 32 and 95 mg g^{-1} DM (Fig. 2c); it was already high by day 15 for leaves incubated at high water nutrients and decreased thereafter, while for leaves incubated at low water nutrient concentration, fungal biomass increased until day 35 and stabilized (Fig. 2c). Fungal biomass was not significantly affected by tree fertilization, water nutrients or their interaction (two-way repeated measures ANOVA, $p = 0.868$, $p = 0.087$ and $p = 0.961$, respectively; Online resource 4).

Table 3 Exponential decomposition rates (k) of leaves originating from unfertilized (NoFert) and fertilized (Fert) trees and incubated in laboratory microcosms under low (LowNP) and high (HighNP) nutrient concentration, standard error (SE) and coefficient of determination of the regression (R^2; $p < 0.001$ in all cases)

Fertilization	Water nutrients	k (day^{-1})	SE	R^2	ANCOVA
NoFert	LowNP	0.0032	0.0005	0.42	A
	HighNP	0.0066	0.0007	0.25	BB
Fert	LowNP	0.0046	0.0007	0.27	AB
	HighNP	0.0073	0.0012	0.03	C

Treatments with the same letter are not significantly different (ANCOVA followed by Fisher's test, $p > 0.050$)

Conidia production by aquatic hyphomycetes

Sporulation rates by aquatic hyphomycetes were already high by the first sampling date for leaves incubated at high water nutrient concentrations (257–274 conidia mg^{-1} DM day^{-1}) and decreased thereafter, while for leaves incubated at low water nutrients sporulation rates remained low throughout (1–59 conidia mg^{-1} DM day^{-1}) (Fig. 2d). Sporulation rates were significantly affected by fertilization, water nutrients, time and their interaction (two-way repeated measures ANOVA, $p = 0.006$, $p < 0.001$, $p = 0.002$ and $p = 0.034$, respectively; Online resource 4). Significant difference among treatments only existed on day 15, with fertilization significantly stimulating sporulation rates on low water nutrient concentration (Tukey's test, $p < 0.001$) but not on high water nutrient concentration ($p = 0.147$), and high water nutrient concentration stimulating sporulation rates on leaves from both fertilization treatments ($p < 0.001$) (Fig. 3d). Leaves from fertilized trees incubated at high water nutrient concentration had significantly higher sporulation rate than leaves from unfertilized trees incubated at low water nutrient concentration on day 15 (Tukey's test, $p < 0.001$).

Fungal assemblages

A total of 15 aquatic hyphomycete species were found across treatments (Table 4). The cumulative number of aquatic hyphomycete species sporulating per treatment varied between 11 and 14 (Table 4). Fertilization, water nutrient concentration and their interaction affected aquatic

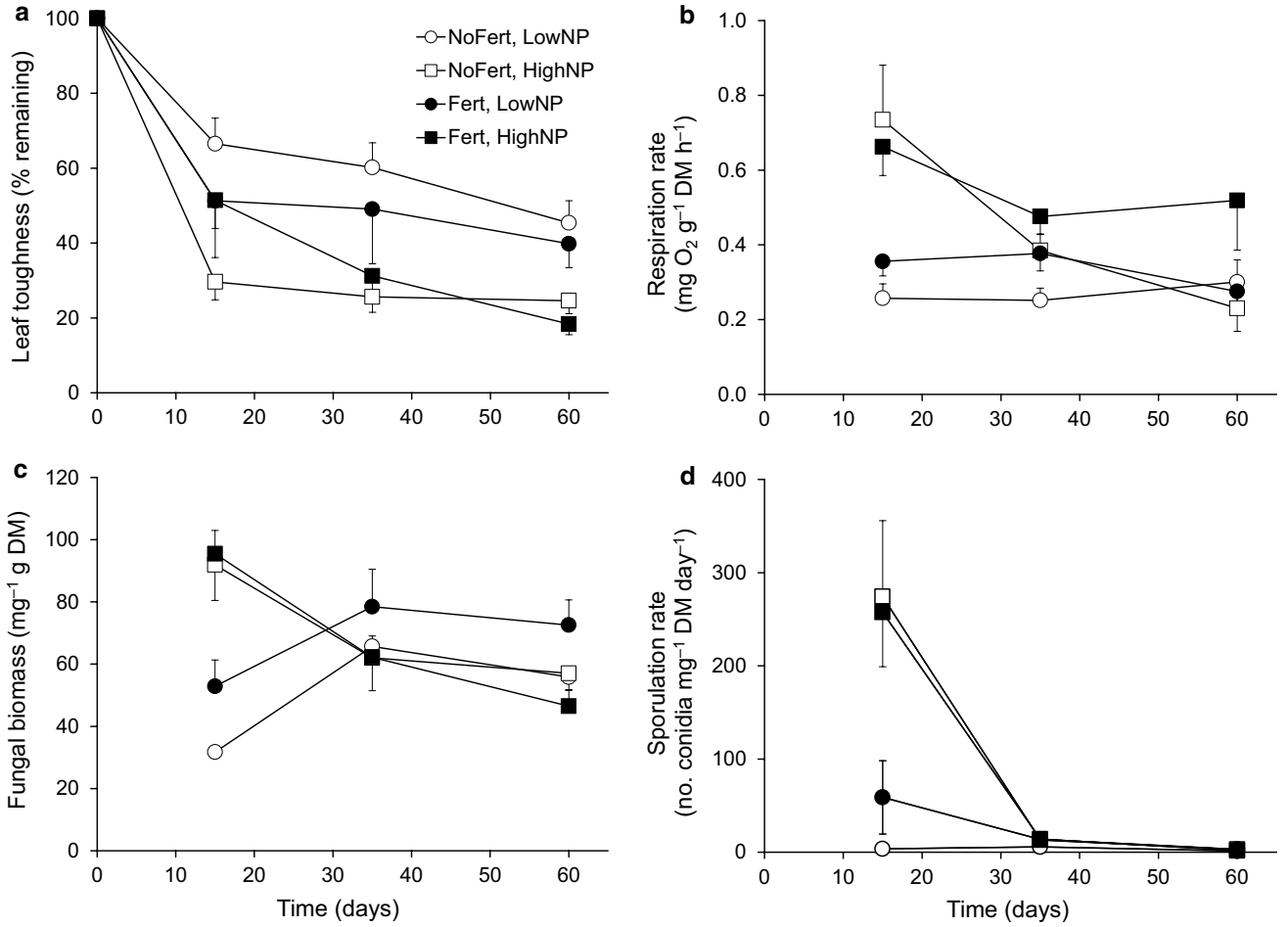

Fig. 2 Leaf toughness remaining (**a**), microbial respiration rate (**b**), fungal biomass (**c**) and sporulation rate by aquatic hyphomycetes (**d**) associated with leaves originating from unfertilized (NoFert) and fertilized (Fert) trees and incubated in laboratory microcosms under low (LowNP, $n = 30$) and high (HighNP, $n = 30$) nutrient concentrations for 15, 35 and 60 days. Values are mean ± SE

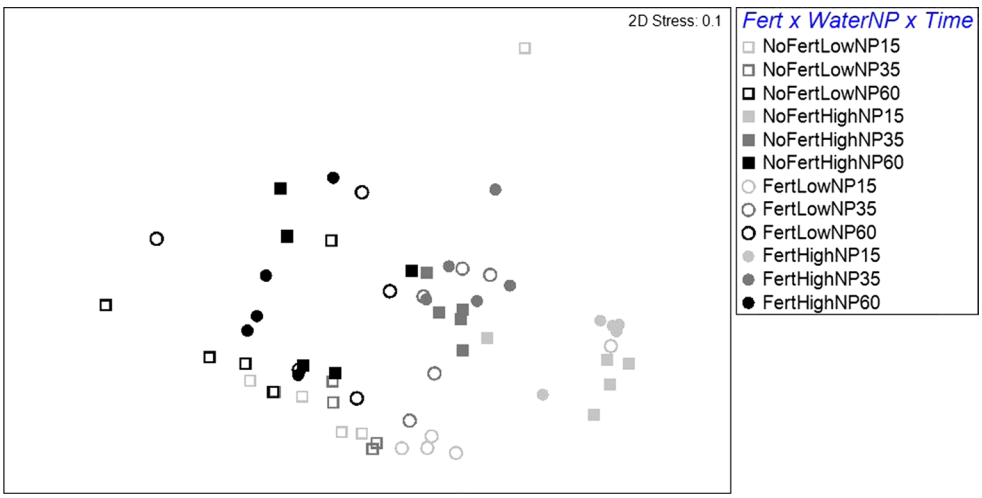

Fig. 3 Ordination (NMDS) of aquatic hyphomycete communities (based on conidial production) associated with leaves originating from unfertilized (NoFert) and fertilized (Fert) trees and incubated in laboratory microcosms under low (LowNP, $n = 30$) and high (HighNP, $n = 30$) nutrient concentrations for 15, 35 and 60 days

Table 4 Relative abundance (%) of aquatic hyphomycete species (based on conidial numbers) associated with leaves originating from unfertilized (NoFert) and fertilized (Fert) trees and incubated in laboratory microcosms under low (LowNP) and high (HighNP) nutrient concentration for 15, 35 and 60 days

Aquatic hyphomycete species	NoFert, LowNP			NoFert, HighNP			Fert, LowNP			Fert, HighNP		
	15	35	60	15	35	60	15	35	60	15	35	60
Alatospora acuminata	13.1	0.1		1.3	3.3	1.0	0.1	5.6	0.9	1.0	11.6	1.2
Clavariopsis aquatica	3.9		0.7	6.0	0.9	0.8	1.1	1.6		4.3	1.8	0.4
Clavatospora longibrachiata		0.9	11.8	4.2	29.5	9.4	1.2	9.9	12.3	1.9	28.6	23.2
Flagellospora curvula			0.6	1.1	0.2	0.3	1.2	0.5		0.1		
Goniopila monticola				0.1		0.2						
Lemonniera terrestris	2.0	1.9	2.3	1.2		0.5	3.1	2.0	5.1	2.6	0.2	1.3
Lunulospora curvula	0.8	0.1			0.2		0.1	0.1				
Tetrachaetum elegans	78.2	94.9	79.8	45.1	39.5	49.8	78.8	45.3	50.1	44.0	24.0	57.1
Tetracladium marchalianum	0.1											
Tricladium chaetocladium	1.2	1.6	1.4	35.1	22.3	31.4	10.1	31.2	18.6	39.3	30.2	8.1
Tricladium splendens	0.1		0.1			0.3	0.4	0.5	0.1	1.0	0.6	5.9
Unidentified tetraradiate sp1			1.9		0.2	0.8	1.2	0.2	2.9	4.2	2.9	2.8
Unidentified tetraradiate sp2		0.5	1.4	5.9	2.6	4.6	2.7	1.6	3.7	1.3	0.1	
Unidentified tetraradiate sp3	0.6				1.3	0.7		1.5	6.3			
Unidentified triradiate sp1						0.2				0.3		
Species richness (no. species treatment^{-1})	9	7	9	9	10	13	11	12	9	11	9	8

hyphomycete species richness (two-way repeated measures ANOVA, $p = 0.001$, $p = 0.001$ and $p = 0.002$, respectively; Online resource 4). Leaves from fertilized trees had significantly higher species richness than leaves from unfertilized trees incubated at low water nutrient concentration (Tukey's test, $p < 0.001$). Also, leaves from unfertilized trees incubated at high water nutrient concentration had higher species richness than leaves incubated at low water nutrients (Tukey's test, $p < 0.001$). Species richness was significantly higher on leaves from fertilized trees incubated at high water nutrient concentration than on leaves from unfertilized trees incubated at low water nutrient concentration (Tukey's test, $p < 0.001$).

In general, three aquatic hyphomycete species, *Tetrachaetum elegans*, *Tricladium chaetocladium* and *Clavatospora longibrachiata*, made up >80% of total conidial production. *T. elegans* was the dominant species in all treatments (42–84%), followed by *T. chaetocladium* (20–30%) and *C. longibrachiata* (4–18%) (Table 4).

Aquatic hyphomycete community structure significantly differed among fertilization (ANOSIM, global $R = 0.13$, $p = 0.004$), water nutrient concentration (global $R = 0.25$, $p = 0.001$) and time (global $R = 0.54$, $p = 0.001$) (Fig. 3). Considering the four treatments altogether, significant differences were observed among all treatments (ANOSIM, $R > 0.17$, $p < 0.028$), except between leaves from fertilized trees at low water nutrients and leaves from unfertilized trees at high water nutrients (ANOSIM, $R = 0.17$, $p = 0.053$; Fig. 3). In particular, communities on leaves from fertilized trees incubated at high water nutrient concentrations significantly differed from those on leaves from unfertilized trees incubated at low water nutrient concentration (ANOSIM, $R = 0.54$, $p = 0.002$; Fig. 3).

Discussion

Here we assessed the direct (water) and indirect (leaf chemistry) effects of nutrient enrichment on leaf litter decomposition and associated microbial decomposers in laboratory microcosms. We showed that microbial decomposers and litter decomposition were stimulated by increases in both dissolved nutrients and leaf N concentration. However, nutrients in water had a stronger effect on litter decomposition and microbial activity.

Soil fertilization increases leaf nutrient concentration

Our hypothesis that soil fertilization influences the chemical composition of the leaves was partially supported, with leaf N concentration being >3× higher in leaves from fertilized than unfertilized trees. It has been noted before that increases in soil N availability due to N fertilization promote leaves with higher nutrient concentration (Bryant et al. 1987; Lawler et al. 1997; Huang et al. 2008; Liu et al. 2010). Other studies have also shown that soil fertilization results in decreased concentration of condensed tannins (Bryant et al. 1987) and that low soil

nutrient availability leads to higher levels of both total phenolics and condensed tannins (Lawler et al. 1997), but these changes were not observed in our case.

Green leaves from fertilized trees had twice the N concentration of senescent leaves suggesting that there was substantial (~50%) resorption of N during senescence. Nutrient resorption is one of the conservation mechanisms that can increase nutrient use efficiency and minimize the dependence of deciduous plants on external nutrient uptake (Norby et al. 2001; Yuan and Chen 2009). Although there is wide variation among species in nutrient resorption efficiency, typically 50% of both the leaf N and P are recycled via resorption (Aerts 1996). Contrary to what we anticipated, no significant N resorption was observed for leaves from unfertilized trees. Generally, plants in infertile habitats have a higher rate of nutrient resorption than those in fertile habitats, which enables them to reuse nutrients more efficiently (Yuan and Chen 2015). However, this hypothesis remains controversial with some studies reporting N and P resorption not linked to plant nutrient status (Chapin and Moilanen 1991; Aerts 1996) and others showing that that resorption depends on soil nutrient concentration (Wright and Westoby 2003; Vergutz et al. 2012). It is plausible that the resorption efficiency decreases with the decrease in the total amount of N in leaves because more energy would be needed to mobilize the small amount of nutrients remaining in leaves. We also observed that in the unfertilized treatment senescence started ~ 20 days earlier than in the fertilized treatment. Maybe this earlier senescence affected nutrient resorption because leaves shed before reabsorption is completed. Despite N resorption only occurring in leaves from fertilized trees, after senescence these leaves still had 2.3× higher N concentration than leaves from unfertilized trees. Effects of fertilization on leaf chemistry can thus prevail after senescence, potentially affecting detrital food webs.

Nitrogen concentration and the C:N ratio did not significantly differ between discs and senescent leaves, and the relative differences in N concentration between fertilization levels were maintained when considering leaf discs. Thus, leaf discs were good surrogates for senescent leaves. Incubation of leaf discs for one week prior to the microcosm experiment induced an increase in N concentration and a decrease in C:N ratio in discs, which was likely due, on one hand, to the leaching of soluble compounds such as polyphenols and simple sugars, and, on the other hand, to microbial colonization and nitrogen immobilization (Gulis and Suberkropp 2003b). The difference in N concentration between fertilization treatments was mitigated during conditioning, but at the beginning of the experiment leaf discs from fertilized trees still had significantly higher N concentration than leaf discs from unfertilized trees.

Water nutrient enrichment stimulates decomposer activities and litter decomposition

In agreement with our prediction, water nutrient enrichment stimulated microbial activity, with microbial respiration, and conidia production peaking earlier and at higher values when dissolved nutrient availability was high. These results were expected and are well documented in laboratory microcosms (Suberkropp 1998; Gulis and Suberkropp 2003a; Ferreira and Chauvet 2011a; Fernandes et al. 2014), whole-stream nutrient addition experiments (Gulis and Suberkropp 2003a; Gulis et al. 2004; Ferreira et al. 2006) and field correlative studies (Rosemond et al. 2002; Gulis et al. 2006).

Microbial decomposers are richer in N and P than the substrates they grow on (Zechmeister-Boltenstern et al. 2015) and thus the microbial growth rate or production is generally limited by the relative low leaf nutrient concentration (Griffiths et al. 2012). Since microbes can retrieve nutrients from both the litter and the water column, an increase in dissolved nutrient availability generally stimulates microbial activity (this study; Gulis and Suberkropp 2003a, b; Ferreira et al. 2006). The activity of microbial decomposers, in particular aquatic hyphomycetes, promotes litter mass loss by multiple pathways. Mineralization of organic C through respiration generally accounts for a large portion of litter mass loss (in the form of CO_2; Hieber and Gessner 2002; Gulis and Suberkropp 2003b; Cornut et al. 2010; Ferreira and Chauvet 2011a, b). The activity of fungal extracellular enzymes promotes litter maceration and facilitates the release of fine particulate organic matter (Arsuffi and Suberkropp 1985; Bärlocher and Sridhar 2014), which is an important food resource for collector and filtering invertebrates. The incorporation of litter C into microbes and the immobilization of dissolved N by microbial biomass renders leaf material a more palatable and nutritious food resource for shredding invertebrates (Gulis and Suberkropp 2003a; Gulis et al. 2006). Finally, the incorporation of litter C into conidia and their subsequent release also manifests in litter mass loss (Gulis and Suberkropp 2003b; Cornut et al. 2010; Ferreira and Chauvet 2011a, b). Thus, human-induced increases in nutrient availability influence the course of decomposition and nutrient cycling within the ecosystem as reported in many studies on nutrient enrichment (e.g. Woodward et al. 2012; Ferreira et al. 2015).

Increases in litter nutrient concentration stimulate decomposer activities and litter decomposition when dissolved nutrient availability is low

In partial agreement with our prediction, leaves from fertilized trees decomposed faster than leaves from unfertilized

trees when dissolved nutrients were low. Litter decomposition is generally faster for high-quality (high N concentration) than for low-quality litter, as microbial colonization and activity are generally higher in the former substrate (Gessner and Chauvet 1994; Gulis and Suberkropp 2003a; Schindler and Gessner 2009; Ferreira et al. 2012; Frainer et al. 2015). However, differences in decomposer activity and litter decomposition between leaves from fertilized and unfertilized trees only when dissolved nutrient availability was low suggest that under low water nutrient concentrations litter microbial decomposers need to return to the litter as a source of N and P (Gulis and Suberkropp 2003a, b; Ferreira et al. 2006; Gulis et al. 2006). When microbial decomposers are exposed to high concentrations of dissolved nutrients, the effects of differences in leaf quality are mitigated, since microbes may satisfy their nutrient requirements from the water. These results indicate a shift in the main source of nutrients for microbial decomposers between the litter (when dissolved nutrient availability is low) and the water (when dissolved nutrient availability is high) and can be interpreted as a strategy of microbial decomposers to save energy since lower energetic investment is needed to retrieve dissolved nutrients versus litter nutrients (Suberkropp and Chauvet 1995; Suberkropp 1998).

The immobilization of N by fungi on leaves from unfertilized trees was higher at high than at low dissolved nutrient concentrations. These results suggest that the magnitude of the response to nutrient enrichment may be driven in part by the initial nutrient concentration of the substrate. Like all living organisms, heterotrophic microbes are fundamentally constrained by the supply of C, N, P, and other essential elements available for growth and reproduction (Suberkropp 1998). Microbial decomposers associated with substrates of low nutrient concentration depend more on external (dissolved) nutrients for growth and reproduction, whereas microbial decomposers colonizing substrates of high nutrient concentration may have a lesser need for external nutrients (Pozo et al. 1998; Gulis et al. 2004).

It is plausible the nutrient enrichment effect on microbial activity and litter decomposition could be stronger than that reported here. We cannot exclude unmeasured changes in leaf discs during microbial inoculation in the week prior to the microcosms experiment. Those changes could include early decomposition with mineralization of leaf C and transfer of leaf C into fungal conidia and would explain why microbial respiration, fungal biomass and conidial production by aquatic hyphomycetes were already high on the first sampling date (day 15) and declined thereafter. Other studies observed the peak in microbial activity on decomposing litter in streams already by day 7 (Gulis et al. 2006; Ferreira and Graça 2016), which was the period we used for discs conditioning.

Fertilization and increases in dissolved nutrients affected aquatic hyphomycete communities

Soil fertilization and water nutrient enrichment affected the aquatic hyphomycete community structure. Conditions with moderate nutrient availability may support higher number of species than conditions with low nutrient availability (Gulis and Suberkropp 2003a; Pascoal et al. 2005; Ferreira et al. 2006), which is consistent with several hypotheses relating ecosystem productivity with diversity (e.g. Tilman et al. 1997). Also, conditions with higher nutrient availability may support higher number of species at a given time due to a release of interspecific competition for resources (Gulis and Suberkropp 2003a; Pereira et al. 2016). Different aquatic hyphomycete communities are likely a consequence of species differing in enzymatic performance (Chandrashekar and Kaveriappa 1988) and chemical composition (Bisht 2013; Grimmett et al. 2013; Danger et al. 2016) and thus having distinct enzymatic capabilities to decompose substrates and distinct nutrient requirements (Bisht 2013). Other studies have shown changes in fungal community structure in woodland streams with nutrient enrichment (Gulis and Suberkropp 2004; Artigas et al. 2008). As detritivores have preferences for certain fungal species (Chung and Suberkropp 2009; Cornut et al. 2015), changes in aquatic hyphomycete community structure with nutrient enrichment likely have consequences for the invertebrate fauna feeding on decaying organic matter and on the higher trophic levels in such streams.

Simultaneous increases in water and litter nutrient concentration stimulate decomposers and litter decomposition: a global change effect

Nutrient availability in streams and soil is likely to keep increasing due to human population growth, intensification of agriculture and other human activities. Our results show that when both litter and water nutrient concentrations increase, microbial activity and leaf decomposition are stimulated. The interaction between increased litter nutrient concentration and increased dissolved nutrient availability was, nevertheless, additive as it was predicted from the individual effects of increases in litter N concentration alone plus those in dissolved nutrient availability alone. The stimulation of microbial activity and litter decomposition by simultaneous increases in litter and water nutrient availability was driven by the increase in dissolved nutrients. Thus, the direct effects of increases in nutrient availability are stronger than those mediated by increases in litter N concentration (indirect effects).

Our results indicate that future increases in nutrient availability (in the range used in this study) may exert a stronger control on microbial activity and litter decomposition

through increases in water nutrients than litter nutrients. Fast decomposition of organic matter entering the streams in autumn under nutrient-rich conditions may lead to food depletion at higher trophic levels later in the year as litter may disappear from the streambed faster (Poff et al. 2002; Rosemond et al. 2015, but see Cross et al. 2006). The rate at which litter decomposes under nutrient-rich conditions may be accelerated by the activities of shredder detritivores that take advantage of the improved litter quality by enhanced microbial activity (e.g. Tonello et al. 2016).

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Author contribution statement MG and VF conceived and designed the experiment. CB and VF performed the experiment and analysed the data. CB, VF, MG and SS drafted the original manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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