The role of aquatic fungi in transformations of organic matter mediated by nutrients

CYNTHIA J. TANT*, AMY D. ROSEMOND*, ANDREW S. MEHRING*, KEVIN A. KUEHN† AND JOHN M. DAVIS*

*Odum School of Ecology, University of Georgia, Athens, GA, U.S.A.
†Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MI, U.S.A.

SUMMARY

1. We assessed the key role of aquatic fungi in modifying coarse particulate organic matter (CPOM) by affecting its breakdown rate, nutrient concentration and conversion to fine particulate organic matter (FPOM). Overall, we hypothesised that fungal-mediated conditioning and breakdown of CPOM would be accelerated when nutrient concentrations are increased and tested the degree to which fungi were critical to CPOM processing and to FPOM production by an invertebrate consumer.

2. We manipulated the presence and absence of fungi, exogenous nutrients [nitrogen (N) and phosphorus (P)] and an invertebrate consumer in a full-factorial laboratory experiment and quantified their effects on CPOM mass loss and nutrient concentration, and the quantity and nutrient concentration of FPOM produced during leaf breakdown.

3. Mass of CPOM lost and the quantity of FPOM produced were highest in nutrient-enriched treatments containing fungal decomposers. Across all treatments, FPOM produced was a constant proportion of CPOM lost (67%). Loss of CPOM due to shredders was highest at fungal biomass values >16 mg g dry mass⁻¹, which occurred with nutrient enrichment.

4. Nitrogen concentration in CPOM increased in treatments with nutrients and fungi, while CPOM P concentration was primarily affected by nutrients. In contrast, FPOM P concentration declined in treatments with fungi and nutrients, suggesting either sequestration via CPOM-associated fungi or preferential assimilation by shredders. Nitrogen in FPOM increased with nutrients, but was unaffected by fungi.

5. Our results indicate that aquatic fungi play a critical role in facilitating energy and nutrient flow through detrital pathways and that their ability to mediate organic matter transformations is significantly influenced by nutrient enrichment.

Keywords: aquatic hyphomycetes, detritus, headwater stream, nitrogen, phosphorus

Introduction

Heterotrophic microorganisms (i.e. bacteria and fungi) are widely recognised for their role in mediating the transfer of energy and nutrients through food webs in many detritus-based ecosystems (Moore et al., 2004; Hagen et al., 2012). Various aquatic ecosystems, from headwater streams to lakes and estuaries, depend on detritus as a dominant or significant source of carbon and nutrients (Wallace et al., 1997; Pace et al., 2004). As the main group of microorganisms associated with coarse particulate organic matter (CPOM) in streams (Findlay et al., 2002), aquatic hyphomycete fungi contribute significantly to resource quality for detrital-feeding consumers (Barlocher, 1985). As a consequence, fungi may also influence downstream food webs via the transformation and conversion of CPOM fractions into fine particulate organic matter (FPOM). Quantifying the role of fungi in organic matter transformations will aid predictions of how alterations in resource availability (e.g. allochthonous carbon, nutrients) potentially affect underlying energy flow and nutrient-cycling processes within detritus-based systems.

Stream ecosystems currently face multiple stressors, including loss of, or changes in, the composition of riparian vegetation and other sources of detritus (Kom-
Fungal impact on detrital transformation

Microbial responses to nutrient enrichment may not only affect CPOM quality directly, but may also affect the quality and quantity of FPOM resulting from consumption by invertebrates. Heterotrophic microorganisms provide the initial pathway by which nutrients are taken up from the water column (Kaushik & Hynes, 1968). Because microorganisms are richer in nutrients than the uncolonised detritus, the concentration of nutrients in the microbial/detrital complex increases with microbial biomass (Stelzer, Hefferman & Likens, 2003), which is critical for the growth of detritivores (Aruffi & Suberkropp, 1985; Barlocher, 1985; Chung & Suberkropp, 2009a,b). Thus, nutrient enrichment may facilitate the increased generation of FPOM via an increase in the CPOM feeding activities of detrital-shredding invertebrates, which may have subsequent effects on consumers that use FPOM as a primary food resource (Pandian & Marian, 1986; Wallace & Webster, 1996).

A large-scale experimental nutrient enrichment of a stream ecosystem resulted in an increased secondary production and flow of nitrogen and phosphorus to detrital consumers, which presumably occurred via microbial nutrient uptake and immobilisation, and subsequent consumption of microbial-colonised detritus (Cross et al., 2007; Davis et al., 2010). Fungi have been implicated as the primary driver of this process, as biomass and production of heterotrophic microorganisms, particularly fungi, increased under nutrient enrichment (Gulis, Suberkropp & Rosemond, 2008; Suberkropp et al., 2010). In addition, nutrient enrichment also resulted in increased ecosystem-scale rates of carbon loss due to increases in microbial respiration and FPOM export (Benstead et al., 2009). Increased FPOM export was probably a result of the positive microbial response to enrichment, which facilitated both microbial- and invertebrate-mediated CPOM mass loss. However, identifying causal mechanisms influencing responses in microbial and invertebrate processes to nutrient enrichment is difficult in catchment-scale field studies. Thus, we tested for and quantified these effects on detrital resources in a laboratory setting.

In the present study, we quantified the role of fungi and their interactions with detrital consumers in facilitating conversion of CPOM to FPOM, which presumably would lead to increased stream-scale FPOM generation under field conditions (Benstead et al., 2009). We manipulated the density of fungal inocula, nutrients and invertebrate consumers in a full-factorial laboratory experiment that quantified the effect of fungal presence on four aspects of organic matter transformation under nutrient enrichment. These experiments tested whether the presence of fungi under ambient or nutrient-enriched conditions differentially influenced the following: (i) the nutrient concentration of CPOM, (ii) CPOM mass loss (i.e. litter breakdown), (iii) the quantity of FPOM produced via invertebrate feeding and (iv) the nutrient concentration of FPOM. Specifically, we predicted that CPOM mass loss and quality would be greatest in the presence of fungi and increased nutrients. In addition, we predicted that FPOM generation and quality would similarly be highest with fungi and increased nutrients. These laboratory studies used biota from the same reference stream at the Coweeta Hydrologic Laboratory (CHL), North Carolina, U.S.A, that were used in the ecosystem-scale studies cited above.

Methods

Experimental design

The experimental design consisted of three factors, each with two levels: fungi reduced (−) or ambient (+), nutrients ambient (−) or moderately enriched (+), and shredders absent (−) or present (+). The treatments established were as follows: (i) −fungi/− nutrients, (ii) −fungi/+ nutrient, (iii) +fungi/− nutrients, (iv) +fungi/+ nutrient, which then were crossed with the presence or absence of the invertebrate shredder, Pycnopsyche sp. (Trichoptera, Limnephilidae). A complete randomised block design was used (n = 5 per in treatment without shredders and n = 15 in treatments with shredders). Additional replicates were included in + shredder treatments to account for variation in individual feeding of Pycnopsyche sp. Microcosms containing sterilised leaf
discs with either ambient or nutrient-enriched [nitrogen (N) and phosphorus (P) at moderate concentrations] stream water were inoculated with a ‘fungi-reduced’ or a ‘fungi-included’ microbial inoculum and allowed to incubate for 14 days. Sterilised leaf discs containing no microbial inoculum (i.e. controls) were also incubated (14 days) in either ambient or nutrient-enriched stream water. After 14 days, a single fifth-instar Pycnopsyche sp. was added to each of the + shredder microcosms (n = 60) and allowed to feed on leaf discs for 72 h. *Pycnopsyche* was selected for this experiment because it is a common leaf-shredding consumer found in streams of temperate deciduous forests (Ross, 1963) and has been implicated as an important driver of organic matter processing (Creed et al., 2009). Additionally, *Pycnopsyche* sp. greatly increased in its relative dominance during the concurrent whole-stream nutrient enrichment experiment, making up the majority of the benthic biomass and secondary production in the enriched stream (Davis et al., 2010).

**Laboratory microcosms**

Red maple (*Acer rubrum*) leaves were collected from Coveeta in the autumn after abscission, returned to the laboratory and allowed to air dry for >2 weeks. Leaves were soaked in deionised water for 48 h, and discs were cut (12 mm diameter). Leaf discs were then dried at 60 °C for 48 h and sterilised with gamma irradiation (20 000 Gy applied over 5 days). We added 20 sterile leaf discs (initial mean ± SE mass = 0.096 ± 0.0004 g) to each microcosm (100-mL plastic beakers) containing 80 mL of either filtered stream water (soluble reactive phosphorus, SRP: 6.8 ± 3.0 μg L⁻¹; dissolved inorganic nitrogen, DIN: 23.2 ± 8.5 μg L⁻¹) or filtered stream water supplemented with N and P at concentrations of 96 μg L⁻¹ SRP and 500 μg L⁻¹ DIN (from stock solutions of NH₄NO₃ and KH₂PO₄ + K₂HPO₄). These concentrations are within the range observed in streams subject to land-use change in the southern Appalachians (Scott et al., 2002) and that were used in the whole-stream nutrient enrichment experiment that inspired these experiments (Rosemond et al., 2008). Microcosms were incubated in a walk-in incubator at 15 °C on a 12/12 h light/dark cycle. Stream water for the study was collected from stream 53 at Coveeta (hereafter referred to as the reference stream) and filtered-sterilised using 0.22-μm Isopore membrane filters (Millipore, Billerica, MA, U.S.A).

To obtain the microbial inocula, red maple leaves at various stages of decay were collected from the reference stream to reflect a representative microbial commu-

To determine mass loss of CPOM and mass of FPOM generated, remaining leaf disc particles (CPOM, >1 mm)
were picked with forceps from each microcosm, and the FPOM suspension (<1 mm) was filtered through a 0.7-
μm glass fibre filter. Coarse particulate organic matter was lyophilised, and FPOM was dried at 60 °C and
weighed. Material was then ground in a ball mill and analysed for C and N concentrations with a Carlo Erba
NA 1500 CHN analyser (Carlo Erba, Milan, Italy). Phosphorus concentration was analysed spectrophotometri-
cally after acid digestion (APHA, 1998).

Litter-associated fungal biomass was estimated from concentrations of ergosterol in plant litter (Gessner,
2005). Ergosterol was extracted from pre-weighed, freeze-dried leaf discs (n = 5 per replicate) in alcoholic
KOH (0.8% KOH in methanol; total extraction volume 10 mL) for 30 min at 80 °C in tightly capped tubes with
constant stirring. The resultant crude extract was partially cleaned by solid phase extraction (Gessner & Sch-
mitt, 1996), and ergosterol was quantified by HPLC. An HPLC (LC-10AT pump and SPD-10A UV-VIS detector,
Shimadzu Scientific Inc., Columbia, MD, U.S.A) was used for separation and analysis of ergosterol. The
mobile phase was HPLC-grade methanol at a flow rate of 1.5 mL min⁻¹. Ergosterol was detected at 282 nm
(retention time ~ 7.5 min) and was identified and quantified based on comparison with ergosterol standards
(Fluka Chemical Co., St. Gallen, Switzerland). Ergosterol concentrations were converted to fungal biomass assuming
an ergosterol concentration of 5.5 μg mg⁻¹ of mycelial dry mass (Gessner & Chauvet, 1993).

Statistical analyses
To test for treatment effects on fungal biomass and litter nutrient concentration prior to shredder feeding, a two-
way analysis of variance (ANOVA) was used to test for main and interactive effects of fungi and nutrients on
fungal biomass (ergosterol) and N and P concentrations of CPOM after the initial 14-day incubation period.
These analyses examined the role of nutrients and fungi in driving initial CPOM quality. A three-way ANOVA
was used to test for the main and interactive effects of nutrients, fungi and shredders in driving mass loss from

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<th>P-value</th>
<th>Nitrogen</th>
<th>F</th>
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<td>Fungi × nutrient</td>
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ns, not significant.

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CPOM and accrual of FPOM. A post-feeding two-way ANOVA was conducted on N and P concentrations of FPOM (from the shredder present treatments only) to test for nutrient and fungal effects on FPOM nutrient concentration. A Tukey-Kramer multiple comparison test was used to test for differences among treatments when significant main or interactive effects of ANOVAs were found.

Relationships between fungal biomass and nutrient concentration of CPOM were also examined with regression analyses. Data were transformed where necessary to improve normality and reduce heteroscedasticity. All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, U.S.A).

Results

Initial fungal biomass

There were significant fungal and nutrient effects, as well as a significant nutrient × fungal interaction on the quantity of fungal biomass that colonised leaf discs during the initial 14-day incubation period (Table 1). Fungal biomass was significantly higher in the fungi + nutrient treatments than in any other treatment (Tukey-Kramer P < 0.0001; Table 2).

Effects of nutrients, fungi and shredders on CPOM mass loss and FPOM production

Mass loss of CPOM was significantly affected by fungi, nutrients and shredders, as well as nutrient × fungi and nutrient × shredder interactions (Table 1). Mass loss was highest in treatments with fungi, nutrients and shredders (Tukey-Kramer P < 0.0001; Fig. 1a). Mass loss in shredder-absent treatments was attributed to leaching and microbial processing of leaf material (e.g. microbial respiration and leaf softening/fragmentation), particularly in the treatments containing both fungi and nutrients. Fungi, nutrients and shredders also significantly

<table>
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<th>Fungi</th>
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<td>Reference</td>
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<td>7.24 (3.23)</td>
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<tr>
<td>Nutrient</td>
<td>5.25 (3.34)</td>
<td>41.75 (6.23)*</td>
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A significant difference in treatments is indicated by an asterisk (ANOVA P < 0.05).
affected the quantity of FPOM produced during the shredder feeding period, with significant interactions among all three factors (Table 1). The amount of FPOM produced within the shredder treatments was significantly higher in the presence of nutrients and in the presence of nutrients + fungi (Tukey–Kramer $P < 0.0001$; Fig. 1b). When compared across all treatments, linear regression analysis found that the mass of FPOM produced was a constant percentage of CPOM lost (67%, $r^2 = 0.93$, SE = 0.003, $P < 0.0001$, $n = 80$).

**Effects of nutrients and fungi on initial CPOM quality**

Both the presence of fungi and exogenous nutrients affected the initial nutrient content of CPOM (Table 1). Across the range in fungal biomass measured in the CPOM treatment combinations prior to shredder feeding, fungal biomass was positively related to CPOM N ($P < 0.0001$; $r^2 = 0.86$; Fig. 2a) and P content ($P = 0.0157$; $r^2 = 0.24$; Fig. 2b). When evaluated categorically based on treatments, CPOM N content was significantly higher in the fungi + nutrient treatments than in all other treatments (Tukey–Kramer $P < 0.0001$; Fig. 3a), but P content was only affected by nutrients (ANOVA, $F_{1,16} = 20.71$, $P = 0.003$) and not fungi (Table 1; Fig. 3b).

**Effects of nutrients and fungi on FPOM quality**

Fungi had no positive effects on FPOM nutrient content and had negative effects on FPOM P content. Nutrient additions resulted in greater FPOM N content (Tukey–
Kramer $P < 0.0001$; Table 1; Fig. 3c), but the increase was not due to fungi (Tukey–Kramer $P = 0.287$). FPOM $P$ content was significantly lower in fungi + nutrient treatments than in any other treatment (Tukey–Kramer $P = 0.037$; Fig. 3d).

Quantitative relationship between fungal biomass and CPOM mass loss

Our experiments allow for examination of how mass loss of CPOM changed across gradients in fungal colonisation in the presence and absence of shredders. Mass loss was greater with shredders present and increased with fungal biomass, which was highest in the range of 20–30 mg g dry mass$^{-1}$ fungal biomass (Fig. 4).

Discussion

Fungi increased the conversion of CPOM to FPOM and had positive effects on the nutrient concentration of CPOM, but had some negative effects on nutrient concentration of FPOM. These data highlight the importance of aquatic hyphomycete fungi in nutrient-mediated enhanced organic matter processing by shredders, supporting previous field-based research at our study sites (Gulis et al., 2008; Suberkropp et al., 2010). Both laboratory and whole-stream studies showed that the contribution and impact of fungal decomposers on detrital processing were increased under nutrient-enriched conditions. Under field conditions, a positive fungal response (e.g. biomass, production, sporulation, etc.) to increased nutrient concentrations has been associated with increased organic matter breakdown rates (Suberkropp & Chauvet, 1995; Gulis & Suberkropp, 2003; Ferreira, Gulis & Graca, 2006). Furthermore, positive impacts of both shredders and nutrients on the downstream export of FPOM have been demonstrated (Wallace et al., 1991; Benstead et al., 2009). Results of our microcosm experiment suggest that, under nutrient enrichment, fungi are explicitly linked to both losses of CPOM and increased production of FPOM due to increased CPOM palatability and consumption by invertebrate shredders.

Fungi and shredders interacted to contribute to the conversion of CPOM to FPOM. Under enriched conditions, CPOM mass loss increased by 23% with fungi alone and by 43% with both fungi and shredders. Our data show that shredders have greater effects at raised fungal biomass (Fig. 4), suggesting that feeding rate increased with higher quality food resources. Similarly, the quantity of FPOM generated by Pycnopsyche nearly doubled in the presence of fungi and increased nutrients as compared to the fungi-reduced treatments. Because fungi, as well as bacteria, often have much lower carbon:nutrient ratios than the substrates that they colonise, their colonisation creates a resource of higher nutritional value for consumers (Cummins & Klug, 1979; Stelzer et al., 2003; but see Danger & Chauvet, 2013). Thus, through immobilisation of nutrients directly from the water column and also through extracellular enzymatic degradation, fungi transform relatively recalcitrant detritus into a more palatable form for detrital consumers (Barlocher, 1985). Shredding invertebrates have been previously cited as important drivers of organic matter transformations of CPOM to FPOM (Wallace et al., 1991), creating a ‘processing chain’ whereby FPOM produced by shredders facilitates collector–gatherer feeding (Short & Maslin, 1977; Heard, 1994). Because the presence of fungi increased FPOM production associated with shredder feeding, our study suggests that fungal conditioning of leaves is an integral first step in this processing chain, which facilitates the role of shredders in increasing the availability of FPOM for other stream consumers. Increased nutrient availability further enhances this chain by decreasing fungal nutrient limitation.

Despite fungi increasing the availability of FPOM, the combined effects of fungi and nutrients did not result in increased FPOM nutrient concentration, but rather decreases in the case of P concentration. Increased resource quality can enhance the nutrient assimilation efficiency of consumers (Pandian & Marian, 1986), and the reduced P concentration of FPOM may have resulted from increased assimilation of P in treatments with
fungi present under raised nutrient conditions. Laboratory studies on *Pycnopsyche* showing increased assimilation of N and P with greater N and P enrichment support this mechanism (J. Davis, unpubl. data). Yoshimura, Gessner & Tockner (2008) reported a similar decrease in P concentration of FPOM generated by the shredder *Gammarus* from three different leaf species. This reduction in FPOM nutrient concentration has the potential to reduce the flow of nutrients to downstream consumers that rely on FPOM (i.e. collector-gatherers). Consumers in our study streams may be primarily P-limited (Cross et al., 2003), so these reductions in FPOM P concentration have the potential to affect consumer production more than the increases in FPOM N concentration. However, the lower P concentration in FPOM we observed may also have been exacerbated by the lack of a flow-through system and conditions of nutrient limitation for FPOM-associated microorganisms even at our increased nutrient concentrations.

Some effects of nutrients on organic matter transformations were also observed in treatments in which fungal biomass was reduced. Even when fungal biomass was reduced, supplemented nutrients still resulted in a 25% increase in CPOM mass loss and a 43% increase in FPOM produced when shredders were present. Nutrient amendments also affected the nutrient concentration of CPOM (N and P) and FPOM (N) in fungal-reduced treatments. These results suggest that increases in detritivore feeding may have been facilitated, at least to some degree, through mechanisms unrelated to fungi, such as increased biomass of leaf-degrading bacteria (i.e. biofilms) or the adsorption of P to leaf surfaces. Observed increases in FPOM N concentration in treatments where fungal biomass was reduced may also have been a result of increased bacterial biomass.

The lack of a measurable effect of fungi under ambient nutrient conditions is probably a result of the short incubation time of this experiment (e.g. 14 days). Because our focus was on nutrient-enriched conditions, peaks in fungal biomass under enriched conditions (which occur well before peaks under ambient conditions) were targeted (Gulis & Suberkropp, 2003). It is possible that fungi may have performed a similar role in nutrient immobilisation under reference conditions if incubated for longer, and effects of similar magnitude may occur, although much more slowly (Cheever, Kratzer & Webster, 2012).

Shredders, such as *Pycnopsyche*, play a major role in CPOM breakdown and the production of FPOM in streams (Wallace et al., 1991; Creed et al., 2009), and both shredder identity and leaf litter species composition may affect the quantity and quality of FPOM in streams (Balseiro & Albarino, 2006; Mehring & Maret, 2011). Our results are limited to a single shredder species transforming detritus from a single leaf species. Red maple and *Pycnopsyche* were chosen for this study due to their prevalence in streams in the eastern U.S.A. Additionally, *Pycnopsyche* has shown a disproportionately positive response to nutrient enrichment compared to other shredder taxa (Davis et al., 2010). The fifth-instar larvae used here were intended as a model for shredders in general, but feed much more slowly than earlier instars of the same species (Chung & Suberkropp, 2009a,b). More work with other leaf and shredder species would further elucidate these interspecific effects. Faecal material from aquatic insects is both a palatable and abundant source of FPOM for collectors; therefore, these transformations of leaf material are important sources of energy and nutrients for these organisms in stream ecosystems (Shepard & Minshall, 1984).

Aquatic fungi are clearly important in detritus-based streams as they provide a highly nutritious food resource for detrital consumers. They may also contribute to food webs downstream as fine particles are transported in the flow. In quantifying the role of fungi in these processes, our study suggests that fungal effects are increased in streams where inorganic nutrient concentrations are raised. In headwater streams of the southern Appalachians, as in many other areas, both farming practices and urban development are major causes of increased nutrient inputs that might be mitigated through management practices such as increasing riparian buffers and educating farmers and homeowners on a more judicious use of fertilisers. An understanding of the complex nature of microbial responses to nutrient enrichment and their cascading effects in stream ecosystems is necessary for proper management and conservation.

**Acknowledgments**

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