Freshwater Biology (2007) **52,** 1762–1772 doi:10.1111/j.1365-2427.2007.01807.x

The quality of organic matter mediates the response of heterotrophic biofilms to phosphorus enrichment of the water column and substratum

MARCELO ARDÓN AND CATHERINE M. PRINGLE
Institute of Ecology, University of Georgia, Athens, GA, U.S.A.

SUMMARY

1. Heterotrophic biofilms are important drivers of community respiration, nutrient cycling and decomposition of organic matter in stream ecosystems. Both organic matter quality and nutrient levels have been shown to affect biofilm biomass and activity individually, but both factors have rarely been manipulated simultaneously.
2. To experimentally manipulate the organic matter quality and phosphorus (P) levels of both the substratum and water column, we first used cellulose cloth as a low-quality organic material and enhanced its quality and P-content by amending the underlying agar with maltose and P, respectively (Experiment I). To manipulate water column P, artificial substrata were incubated in low- and high-P sites of a whole-stream P-enrichment in lowland Costa Rica.
3. Results from Experiment I suggest that heterotrophic biofilm respiration on cellulose cloth is co-limited by carbon (C) and P. Biofilm respiration responded in an additive manner to combined effects of maltose and P-enrichment of water column and synergistically to maltose and high-P in substrata.
4. As decomposing organic matter that supports heterotrophic biofilms varies naturally in its labile C content along with other physical and chemical properties, we conducted a second experiment (Experiment II) in which we amended leaf discs from two species (*Trema integerrima*, a labile C source and *Zygia longifolia*, a recalcitrant C source) with maltose. We incubated the substrata in low- and high-P sites of the P-enrichment stream.
5. Results from Experiment II indicate that biofilm respiration on a labile C source (*Trema*) was not C-limited, while biofilm respiration on a recalcitrant C source (*Zygia*) was C-limited. Phosphorus stimulated the biofilm respiration and breakdown rate on *Trema*, but not on *Zygia*, supporting the hypothesis that the stimulatory effect of P-enrichment is dependent on the availability of labile C in decomposing leaves.
6. Our results suggest that the interactive effects of organic matter quality and nutrient loading of streams can significantly increase microbial biofilm activity, potentially altering the trophic base of stream food webs. Researchers should consider both the organic matter quality and the enrichment of both water column and substrata to better predict the effects of anthropogenic nutrient loading to stream the ecosystems.

Keywords: Costa Rica, decomposition, fungi, microbial respiration, nutrients

Introduction

Heterotrophic biofilms, composed primarily of fungi and bacteria, are important drivers of community respiration, nutrient cycling and organic matter

Correspondence: Marcelo Ardón, Department of Biology, Duke University, P.O. Box 90338, Durham, NC 27708, U.S.A.
E-mail: mla5@duke.edu

© 2007 The Authors, Journal compilation © 2007 Blackwell Publishing Ltd

decomposition in stream ecosystems (Mulholland *et al*., 1984; Tank & Webster, 1998; Battin *et al*., 2003; Tank & Dodds, 2003). Because of the important role of heterotrophic biofilms in transforming and transferring allochthonous carbon (C), increases in their biomass can stimulate the abundance and biomass of higher trophic levels (i.e. invertebrate consumers; Wilcox *et al*., 2005). Previous studies have shown that biomass and activity of heterotrophic biofilms are influenced by the quality of organic matter (McArthur, Marzolf & Urban, 1985; Hedin, 1990; Meyer, 1994; Koetsier, McArthur & Leff, 1997; Leff, 2000) and water column nutrient levels (Tank & Webster, 1998; Grattan & Suberkropp, 2001; Tank & Dodds, 2003). Effects of organic matter quality and water column nutrient levels on microbial biofilms have primarily been studied independently.

Previous studies that have addressed how biofilms are affected by both water column nutrients and organic matter quality have incubated leaves and/or wood from different species in stream nutrient enrichment experiments (Royer & Minshall, 2001; Stelzer, Heffernan & Likens, 2003; Gulis *et al*., 2004; Ardón, Stallcup & Pringle, 2006; Greenwood *et al*., 2006; Stallcup, Ardón & Pringle, 2006). Nitrogen (N) and phosphorus (P) enrichment of the water stimulated the fungal and bacterial biomass and microbial respiration on low quality wood (high C : N ratio) more than on high quality leaves (with low C : N, Stelzer *et al*., 2003; Gulis *et al*., 2004). Similarly, Greenwood *et al*. (2006) reported that breakdown of low quality (high C : N) rhododendron (*Rhododendron maximum* L.) leaves tended to respond more strongly to N and P enrichment than high quality (low C : N) red maple (*Acer rubrum* L.) leaves. In contrast, Ardón *et al*. (2006) reported that microbial respiration and fungal biomass responded more strongly to P-enrichment on litterbags of a high-quality species (low lignin and cellulose) than on litterbags of low-quality species (high lignin and cellulose).

Because past studies have relied on natural variations among species to manipulate organic matter quality, their conclusions have been limited by confounding factors because of correlations among the chemical and physical parameters that determine quality. For example, there is a high degree of correlation between the concentrations of labile forms of C and leaf N content (Webster & Benfield, 1986), making it difficult to isolate their individual effects. Agar-diffusing artificial substrata can provide an alternative to using natural variations among species by allowing the manipulation of concentrations of labile forms of C and nutrients. We used agar-diffusing artificial substrata to experimentally examine the interactive effects of organic matter quality and water column nutrient levels on biofilm respiration. Agar-diffusing substrata are also ideal experimental units to examine how nutrient enrichment of substratum or water column can interact with organic matter quality to determine heterotrophic biofilm activity.

The simultaneous effects of organic matter quality and nutrients levels (in both water column and substratum) can be important in determining biofilm activity, given that biofilms obtain nutrients from both organic matter and overlying water (Suberkropp, 1998). Stream benthic habitats are mosaics of microenvironments that can differ in chemical and physical nature (Pringle *et al*., 1988). Numerous studies have shown substantial changes in concentrations and availability of nutrients and C over short distances in streams (Meyer & Tate, 1983; Hedin, 1990; Dent & Grimm, 1999; Dent, Grimm & Fisher, 2001). Patches of nutrient- and C-rich sediments develop because of localized accumulations of organic matter, seepage of solute-rich groundwater and nutrient excretion from consumers, which can stimulate growth and diversity of algal and bacterial communities (Pringle & Bowers, 1984; Pringle *et al*., 1986; Crocker & Meyer, 1987; Meyer, Edwards & Risley, 1987; Pringle, 1990).

Here, we experimentally examined the effects of P-enrichment (of the substratum and the water) and organic matter quality on biofilm respiration and leaf breakdown rates. We conducted two experiments: first we used cellulose cloth as a low-quality organic material and enhanced its quality and nutrient content by amending the underlying agar with maltose (high-quality C source) and P, respectively. Artificial substrata were incubated in low- and high-P sites of a whole-stream P enrichment experiment. Because decomposing organic matter that supports heterotrophic biofilms varies naturally in the labile C content along with other physical and chemical properties, we then conducted a second experiment, amending the leaf discs from two species (*Trema integerrima*, a labile C source; *Zygia longifolia*, a

recalcitrant C source) with maltose and incubated them in low- and high-P sites of the P-enrichment stream.

Methods

Site description

This study was conducted at La Selva Biological Station, Costa Rica (10°26′N, 84°01′W). The 1536 ha reserve is the lowland terminus of the last protected unbroken biological corridor, spanning the full altitudinal range on the Caribbean slope of Central America. La Selva receives 4000 mm of rain a year, with more than 400 mm a month from May to December (Sanford *et al.*, 1994).

Because of dense canopy cover (>90%), streams are heavily shaded, resulting in predominantly detritus-based food webs (Pringle *et al.*, 1993). Streams at La Selva exhibit natural variation in water column P concentration [5–350 μg soluble reactive P (SRP) L^{-1}] and other solutes (Mg, Fe, Na, Cl and SO_4) because of inputs of solute-rich groundwater (Pringle *et al.*, 1993). This study was conducted in the Carapa, a first-order stream that drains into the Sura River and does not receive the solute-rich groundwater. We have been experimentally enriching a 100-m reach of the Carapa with phosphoric acid since July 1998 as part of a larger study examining the effects of solute-rich groundwater inputs on ecosystem processes (Ramírez, Pringle & Molina, 2003; Ardón *et al.*, 2006; Ramírez & Pringle, 2006). Phosphoric acid has been added to increase P concentrations from <10 μg to a target concentration of 300 μg SRP L^{-1}, which is at the high end of P levels exhibited by streams receiving solute-rich groundwater (Pringle & Triska, 1991). A Mariotte bottle (carboy) is used to add phosphoric acid continuously, with concentration and drip rate adjusted according to stream discharge. Discharge was measured bi-weekly, and water samples were taken weekly at one site upstream and three sites downstream from the site of P addition (10, 50 and 100 m) for SRP analysis (APHA, 1998). A previous study indicated that NO_3 and NH_4 concentrations do not change significantly the downstream from the point of P-enrichment (Stallcup *et al.*, 2006), thus we did not collect samples for NO3 and NH4 analysis from downstream of the enrichment. The experimental method was described in greater detail in previous studies (Ramírez *et al.*, 2003; Ardón *et al.*, 2006; Stallcup *et al.*, 2006).

Artificial substrata

Artificial substrata were constructed by modifying techniques developed to examine nutrient-limitation of heterotrophic biofilms (Tank & Webster, 1998; Tank & Dodds, 2003; Tank, Bernot & Rosi-Marshall, 2006). The substrata consisted of 30-mL plastic containers filled with a 2% (weight by volume) agar solution. We conducted two separate experiments in which the artificial substrata were placed above and below the site of P-enrichment.

In Experiment I, conducted from 2 to 18 March, 2005, we examined the interactions between presence/absence of high-quality C and P both in water and substratum. We used an artificial standardized low-quality material (cellulose cloth, American Livestock Supply Inc., Madison, WI, U.S.A.) as an organic site for attachment of fungi and bacteria. The cellulose cloths were placed to cover the agar completely and secured with a tight fitting snap-on cap exposing a 4-cm diameter circle on the top of the containers.

We manipulated organic matter quality (presence or absence of maltose in the agar), substratum P (present or absent) and water column P (low or high). For the substratum P treatment, we added 0.5 M of KH_2PO_4 to the agar (Tank & Dodds, 2003). To manipulate quality, we added maltose to the agar at a concentration of 4% (weight by volume) because it is a readily available simple sugar that can be easily broken down by heterotrophic biofilms (Canhoto & Graça, 1999). Four replicates of each treatment were secured to L-shaped plastic beams and attached to the bottom of the stream above and 10 m below the site of P-enrichment (4 treatments × 2 sites × 4 replicates = 32 substrata).

Substrata were collected on day 16, as previous experiments had shown that 16 days were the time to maximum microbial respiration on cellulose cloths (M. Ardón, unpublished data). Substrata were brought to the laboratory, and biofilm respiration on the cellulose cloths was measured on the same day of collection. We placed the entire cellulose cloth in respiration chambers (26 mL) filled with stream water from the site where the substrata had been collected. Changes in dissolved oxygen concentration were measured every 5 min for 30 min using a YSI Model

58 dissolved oxygen metre with a self-stirring probe (Gulis & Suberkropp, 2003). Chambers containing only stream water were used as controls. Oxygen consumption is expressed as mg O_2 per gram of ash-free dry mass (AFDM) per hour. AFDM was determined by drying each disc at 70 °C for 24 h, weighing, burning at 500 °C for 1 h and re-weighing. A 1/4 sub-section of each disc was stored in HPLC grade methanol to be used for ergosterol determination using standard methods (Suberkropp & Weyers, 1996).

In Experiment II, conducted from 3–19 July 2005, we explored whether increasing the quality of different natural leaves, which differed in C availability, alters the response of biofilms to P enrichment. We modified the quality of the substrata by adding maltose to the agar as a high-quality C source. We used leaf discs from two different tree species and placed them on top of the substrata. Cellulose cloths were used as controls. Two species of common riparian trees were selected: *Trema integerrima* [(Beurl) Standl (family Ulmaceae; hereafter referred to as *Trema*)], and *Zygia longifolia* [(Humb. & Bonpl. Ex Willd.) Britton & Rose (family Fabaceae; hereafter referred to as *Zygia*)]. Leaves of *Trema* have low concentrations of lignin, tannins and phenolic compounds, and exhibit fast breakdown rates. Leaves of *Zygia* have high concentrations of phenolic compounds and exhibit slow breakdown rates (Table 1). Both species have similar initial P content (Table 1). The leaf discs were held in place with a tight fitting snap-on cap exposing a 4-cm diameter circle of leaf material on the top of the containers. For each of the three treatments (*Trema*, *Zygia* and cellulose cloths), half of the substrata received the agar amended with 4% maltose as a source of high-quality C, while the other half received agar only.

Artificial substrata were secured to L-shaped plastic beams in groups of six (one replicate per treatment), as follows: *Trema* without maltose, *Trema* with maltose, *Zygia* without maltose, *Zygia* with maltose, cellulose without maltose and cellulose with maltose. The placement of the treatments was randomly assigned within individual plastic beams, which were secured to the bottom of the stream using stakes above and 10 m below the site of P-enrichment. Because of the rapid breakdown of *Trema* leaves, we collected the substrata with *Trema* on days 2, 4 and 8 (3 collection dates × 2 maltose treatments × 2 P treatments × 4 replicates = 48 substrata). We collected substrata with *Zygia* and cellulose cloth discs on days 2, 4, 8 and 16 (4 dates × 2 maltose treatment × 2 P treatments × 4 replicates = 64 substrata each). Samples were taken back to the laboratory to measure the biofilm respiration and AFDM remaining as described above.

To examine the rate of maltose release from agar to the water, we used a semi-quantitative colorimetric assay for dissolved sugars (Benedict's reagent). To examine if the agar itself could serve as a C source, we conducted a preliminary experiment in which we compared respiration after 16 days on cellulose cloths secured on the top of plastic containers without agar to cellulose cloths on containers with agar.

Statistical analyses

We analysed the data from Experiment I using three-way analysis of variance (ANOVA), with substratum P, maltose and water column P as the main effects, followed by *post hoc* comparison of least squares means (Tukey's honestly significant difference). Respiration data were log-transformed to meet assumptions of equal variances and normality. In Experiment II, we calculated the breakdown rates as the negative slope of the linear regression of natural log of percent AFDM remaining versus days in the stream (negative exponential model; Benfield, 1996). Analysis of covariance (ANCOVA) was used to compare the breakdown rates among treatments. Repeated measures ANOVA was used to test for effects of maltose and P on biofilm respiration within each substratum type (*Trema*, *Zygia* and cellulose). Respiration data were log transformed to meet assumptions

Table 1 Initial leaf litter chemistry (percent dry mass) of two common riparian species in La Selva Biological Station, Costa Rica. Breakdown rates are from Ardón *et al.* (2006)

Species	Lignin (% DM)	Cellulose (% DM)	Hemicell (% DM)	C (% DM)	N (% DM)	P (% DM)	Breakdown rate (k day^{-1})
Trema integerrima	1.25	11.51	8.88	34.09	1.35	0.074	0.066
Zygia longifolia	21.36	21.74	19.55	46.60	2.07	0.071	0.005

of equal variances and normality. Analyses were conducted by using the JMP 5.0.1 statistical software (SAS Institute, Cary, NC, U.S.A.).

Results

Whole-stream P enrichment of the Carapa during the experiment was characterized by temporary shifts in P-concentration because of the changes in discharge and variable rates of P-release from the Mariotte bottle (Table 2). We were able to maintain the high levels of P-enrichment through the duration of the experiments (Table 2). Concentrations of nitrate and ammonium, temperature, conductivity and pH were similar between the two experiments (Table 2).

We found a constant rate of maltose release from the agar during the first 21 days, followed by a rapid decline. This suggests that biofilms on substrata with maltose amendments received a constant source of labile C throughout the duration of the experiments. We found significantly higher respiration on substrata with agar than on the substrata without agar (mean respiration with agar = 0.41 ± 0.90 mg O_2 g $AFDM^{-1}$, mean respiration without agar = 0.13 ± 0.92 mg O_2 g $AFDM^{-1}$; $F = 7.96$, $P < 0.05$), indicating that just the agar itself (without P or maltose amendments) could serve as a C source for biofilms.

In Experiment I, the experimental treatments supported different rates of biofilm respiration ($F = 12.0$, $P < 0.001$, Fig. 1). Microbial respiration was stimulated by all three treatments (P-enrichment of overlying water, addition of P to the substratum and the addition of maltose to substratum; Table 3). While the individual treatments stimulated respiration, there was no significant difference (Fig. 1). When maltose and P were added together, the biofilms' response was much stronger (*post hoc* Tukey's HSD $P < 0.05$, Fig. 1). We also observed additive effects between maltose and P treatments. When maltose was added in combination with P-enrichment of the water, biofilm respiration was similar to the sum of the individual treatments, indicating an additive response (Fig. 1). Biofilm respiration responded more strongly to maltose + substratum-P amendments, than to maltose + water column P amendments (Fig. 1). Respiration in response to both maltose and substratum-P was higher than the sum of the individual treatments, indicating a synergistic response.

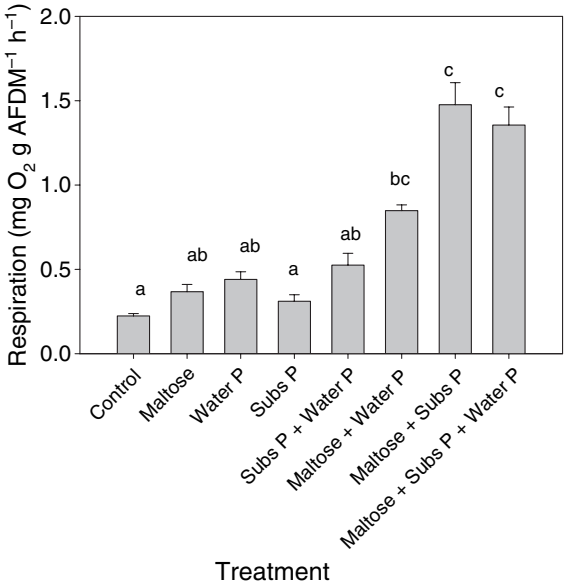

Fig. 1 Mean (±1 SE) biofilm respiration rate on artificial substrata after 16 days in the Carapa stream. Different letters denote significant difference from *post hoc* comparison of least squares (Tukey's honestly significant difference). Subs *P*, substrate phosphorus.

Table 2 Mean and range (in parentheses) of physical and chemical characteristics of the Carapa study stream during the study periods. Reference reach is 10-m upstream of the site of phosphorus-enrichment, while enrichment is 10-m downstream

	Experiment I		Experiment II	
	Reference	Enrichment	Reference	Enrichment
Discharge (L s^{-1})	1.76 (0.82–1.86)		0.49 (0.22–0.62)	
pH	5.57 (5.18–6.48)	5.53 (5.16–6.11)	5.25 (5.01–5.85)	5.15 (5.02–5.47)
Conductivity (μS)	17.8 (14.6–24.6)	17.2 (12.8–24.8)	14.3 (13.9–14.6)	15.7 (13.8–18.8)
Temperature (°C)	24.5 (24.4–24.6)	24.5 (24.5–24.6)	25.4 (25.4–25.6)	25.5 (25.5–25.6)
SRP (μg L^{-1})	3 (3–5)	2781 (516–4419)	1 (0.3–2.3)	993 (63–3428)
NO_3 – N (μg L^{-1})	177 (172–185)		140 (137–143)	
NH_4 – N (μg L^{-1})	3.1 (0–12)		Below detection limit	

Table 3 *F*-ratios for three-way analysis of variance for Experiment I

Factor	DF	*F* ratio
Water P	1	11.7**
Subs P	1	15.5***
Maltose	1	43.3***
Water P * subs P	1	4.1
Water P * maltose	1	0.2
Subs P * maltose	1	6.8*
Water P * subs P * maltose	1	2.3

P, phosphorus; subs, substratum. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In Experiment II, breakdown rates were fastest for *Trema*, and slowest for cellulose cloth (Fig. 2). P-enrichment in the water significantly accelerated the breakdown rate of *Trema* with ($F = 12.49$, $P < 0.005$) and without maltose ($F = 14.88$, $P < 0.005$; Fig. 2a), but maltose had no significant effect on the breakdown rate. Maltose and P-enrichment tended to accelerate the breakdown rate of *Zygia* discs and cellulose cloth, although treatment differences were not significantly different (Fig. 2b,c).

Biofilm respiration on *Trema* leaf discs increased with time in the stream and was stimulated by P-enrichment in the water (date $F = 26.88$, $P < 0.001$; date*P $F = 3.91$, $P < 0.05$; Fig. 3a). Maltose amendments did not change the response to water column P (date*P*maltose $F = 2.16$, $P = 0.16$; Fig. 3a). Respiration rates on *Zygia* and cellulose cloth were much lower than on *Trema*. Respiration rates on *Zygia* increased over time, and were stimulated by the presence of maltose (date $F = 4.72$, $P < 0.05$, date*-maltose $F = 3.45$, $P < 0.05$; Fig. 3b). However, maltose

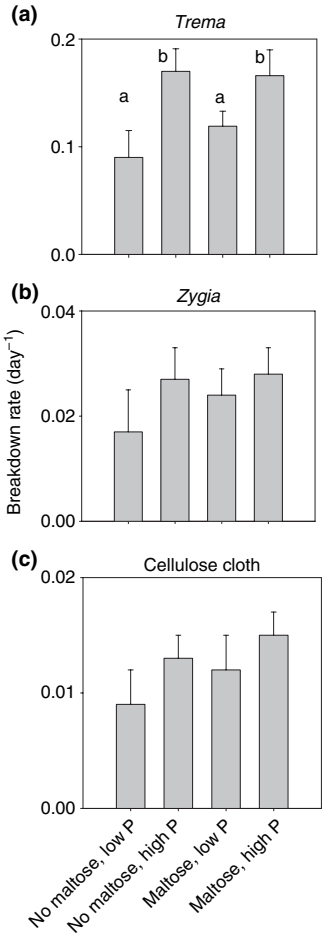

Fig. 2 Leaf breakdown rates (day^{-1}) for (a) *Trema* leaf discs; (b) *Zygia* leaf discs; and (c) cellulose cloth on artificial substrata. Error bars represent ± 1 SE.

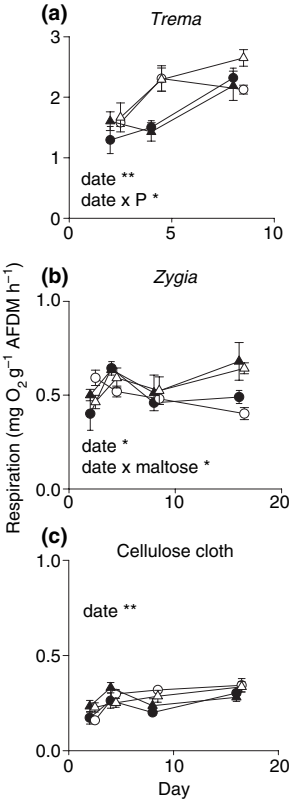

Fig. 3 Mean (±1 SE) microbial respiration on (a) *Trema* leaf discs; (b) *Zygia* leaf discs; and (c) cellulose cloth on artificial substrata. Treatment symbols: no maltose and low phosphorus (P) (black circles); no maltose and high P (white circles); maltose and low P (black triangles); maltose and high-P (white triangles). Factors on graphs are from repeated measures ANOVA. $^{*}P < 0.05$, $^{**}P < 0.001$. Note different scales on axes. Symbols are offset by 0.5 day for presentation purposes.

did not alter the response of biofilm respiration to high-P in the water on *Zygia* (date*P*maltose $F = 2.11$, $P = 0.16$; Fig. 3b). Respiration on cellulose cloth increased over time (date $F = 13.28$, $P < 0.001$), but was not significantly affected by high-P in the water, addition of maltose to the agar, or the interaction between the two (date*P $F = 2.03$, $P = 0.17$; date*maltose $F = 1.15$, $P = 0.37$; date*P*maltose $F = 1.48$, $P = 0.27$; Fig. 3).

Discussion

Our results provide experimental evidence to support the hypothesis that organic matter quality can determine the magnitude of stimulation of biofilm respiration in streams in response to P enrichment of the water and substratum. We found evidence for both C and P limitation of biofilm respiration and leaf breakdown rate. Combination of labile C amendments and P-enrichment produced an additive response when P was added to the water, and a synergistic response when P was added to the substratum. Our results also suggest that heterotrophic biofilms on leaves that contain labile C can become P-limited, whereas biofilms on leaves that have high concentrations of recalcitrant C are C-limited and are not stimulated by P-enrichment of the water column.

Evidence for C-limitation of heterotrophic biofilms

Positive responses to maltose amendments in both experiments suggest that heterotrophic biofilms are C-limited in our study stream. Maltose amendments stimulated the biofilm respiration when compared with controls in both experiments. In Experiment II, the quality of natural leaves appeared to determine the biofilm response to maltose amendments (Fig. 3). Maltose increased biofilm respiration rate on *Zygia* leaf discs (low-quality), but did not affect the respiration or breakdown in *Trema* leaf discs (high-quality; Fig. 3). We did not find an increase in the breakdown rate of *Zygia*, suggesting that microbial biofilms increased their respiration rates by taking advantage of labile dissolved organic C (DOC) from maltose amendments, without increasing their use of leaf litter C. This is not unexpected as *Zygia* leaves have high concentrations of cellulose and lignin, which can slow down microbially mediated decomposition (Ardón *et al.*, 2006).

We hypothesize that the delayed (i.e. day 16) response of microbial respiration to P-enrichment on *Zygia* leaf discs was due in part to initial presence of secondary compounds in natural leaves. Secondary compounds extracted from red maple leaves have been shown to inhibit the bacterial populations on artificial substrata (McNamara & Leff, 2004). In a previous study, we found that the majority of tannins and phenolics were leached out from *Zygia* leaves in approximately 7 days (Ardón, 2006). The initial presence of the secondary compounds likely prevented biofilms on *Zygia* leaf discs from responding to maltose amendments earlier in the incubation period. When the secondary compounds were leached from the leaves around day 8, biofilms likely became C-limited because of high concentrations of cellulose and lignin in *Zygia* leaves. It is probable that if we had run the experiment for a longer period, we would have seen a stronger biofilm response to maltose addition on *Zygia* leaf discs.

Even though we did not measure the concentration of DOC in our study stream, mean concentration in a nearby similar stream (Taconazo) is 1.7 mg L^{-1} (W.H. McDowell, C.M. Pringle and D. Genereux, unpublished data), suggesting that DOC is relatively low in these streams. Stimulation of microbial activity because of maltose amendments would therefore be expected in streams at La Selva. Our results agree with a previous study that showed increased bacterial biomass on artificial substrata with glucose amendments in an Ohio stream (Olapade & Leff, 2005). Similarly, a whole-stream addition of labile DOC (potassium acetate) at Hubbard Brook Long-Term Ecological Research Site led to increases in microbial respiration (Bernhardt & Likens, 2002). A similar study at Coweeta Hydrologic Laboratory found that whole-stream enrichment with labile DOC (dextrose) stimulated the microbial respiration on red maple leaves (Wilcox *et al.*, 2005).

Effects of organic matter quality and P-enrichment of substratum and water column on biofilm respiration

Our results indicate that the availability of labile C magnifies the stimulatory effect of P enrichment on biofilm activity. The magnitude of stimulation to both P treatments was much higher when maltose was present. We found an additive response of biofilm respiration to combined influences of maltose amend-

ments and high-P in the water, and a synergistic response to maltose and substratum-derived P on biofilm respiration (Fig. 1). It is possible that the synergistic response of biofilms to maltose and substratum-derived P was primarily because biofilms received higher concentrations of P from the enrichment of the substratum, than from enrichment of overlying water. However, respiration in the substratum-P treatment was lower than respiration in water-P treatment (Fig. 1). This suggests that it was the combination of labile C and localized high concentrations of P in the substratum-P treatment which led to the synergistic response.

Biofilms in our experiments responded differently to nutrient enrichment of substratum and water. Upwelling of solute-rich groundwater, nutrients from consumer excretion in larval retreats and inputs of nutrient rich leaves can all create patches of nutrient-rich substrata for biofilms (Pringle & Bowers, 1984; Crocker & Meyer, 1987; Meyer *et al*., 1987; Pringle *et al*., 1988; Pringle, 1990). Our results suggest that high P levels in substrata and the availability of labile C can lead to hot spots of high heterotrophic biofilm activity. Previous research showed that algae also respond differently to increases in nutrients coming from substratum or overlying water, and that this can be an important mechanism determining algal diversity (Pringle, 1990). Future research should examine whether nutrients from different sources also affects the diversity of fungi and bacteria comprising heterotrophic biofilms.

To determine if the respiration response was driven by fungi, as has been demonstrated for other streams (Stelzer *et al*., 2003; Gulis *et al*., 2004), we measured ergosterol on subsamples of cellulose cloths from Experiment I. Contrary to what we expected, all samples were below the detection limit. We previously reported high concentrations of ergosterol in decomposing leaves in this stream (Ardón *et al*., 2006; Stallcup *et al*., 2006). We believe the low ergosterol concentrations might have been due to the low quality of the cellulose substrata. Because of the low ergosterol concentrations in Experiment I, we did not collect samples for ergosterol analysis from Experiment II. The low ergosterol concentrations in Experiment I suggest that the respiration response was mostly driven by bacteria. However, we did not measure bacterial biomass on the leaf discs. Algae are also part of biofilms, so it is possible that the respiration response might have been driven by algae. We think this is unlikely, however, because previous experiments have shown low levels of algal biomass in this stream (mean chl a 1.5 mg m^{-2}, A. Ramírez and C.M. Pringle, unpublished data; Pringle *et al*., 1986).

Biofilms on *Trema* leaf discs were not C-limited and thus did not respond to increased labile DOC from maltose amendments. We did not observe a significant interaction between P-enrichment of the water and maltose amendments in *Trema*. P-enrichment of the water stimulated the microbial respiration and leaf breakdown of *Trema* leaf discs, while maltose had no effect. The increase in respiration and breakdown rate on *Trema* leaves, in response to P-enrichment of the overlying water, supports our previous work that the availability of labile C can increase the response to P-enrichment (Ardón *et al*., 2006). Previous studies have similarly shown that the presence of labile DOC (leaf leachate or glucose) can increase N uptake by microbial biofilms in sediments (Bernhardt & Likens, 2002; Sobczak, Findlay & Dye, 2003).

Effectiveness of artificial substrata as bioassays

Major considerations when using artificial substrata to examine heterotrophic biofilms include: variability in the rate of nutrient and C supply, selection of type of substratum for microbial attachment and experimental artefacts because of unmodified agar serving as a C source. Issues regarding the rate and duration of nutrient supply using agar substrates have been previously discussed (Pringle, 1987; Pringle & Triska, 1996; Tank & Dodds, 2003). Our results clearly indicate that the type of substratum for microbial attachment can have important effects on biofilm response to C and nutrient manipulations (Figs 2 & 3).

Cellulose cloths provided a standardized low-quality material, as evidenced by similar respiration rates on the control treatments between the two experiments (Figs 1 & 3). The lack of treatment separation in Experiment II on cellulose cloths may be due in part to lower discharge during Experiment II, which led to increased sediment accrual on the cellulose cloths and increased colonization by larval chironomids (Diptera: Chironomidae). Inorganic matter on cellulose cloths was lower in Experiment I (mean = 9 ± 3 mg inorganic matter per disc) than in Experiment II (mean = 44 ± 2.3 mg inorganic matter per disc, $F = 120$, $P < 0.0001$). We also observed

higher, but not significant, Chironomidae abundance on cellulose cloths in Experiment II (mean = 7 ± 4 individuals per cellulose cloth) than in Experiment I (mean = 3.2 ± 2 individuals per cellulose cloth, $F = 2.38$, $P > 0.05$), which could have decreased the microbial biomass through their feeding activities. We did not find significant differences among treatments within each of the experiments in Chironomidae abundance.

Higher respiration on substrata with agar indicates that unmodified agar can in fact serve as a C source. While this should be considered for future experiments using artificial substrata, we do not think that it significantly biased our results. Our overall conclusions do not change even when we subtract the respiration difference between our agar and no agar treatments. To minimize the possible experimental artefacts associated with variation in the quality of the agar itself, we suggest using the same lot of a refined brand of commercial agar for experiments. Previous research has suggested that using different lots of agar in the substratum may lead to different amounts of P leaching from the substrata (Pringle, 1987).

In conclusion, our results provide experimental evidence to support the hypothesis that organic matter quality can mediate the magnitude of stimulation of biofilm respiration in response to P enrichment of water and substratum in streams. Factors that might alter the quality of organic matter inputs into streams, like organic matter loading from untreated or partially treated sewage, could magnify the response of heterotrophic biofilms to nutrient loading. Future research should consider various sources of nutrients (water and substratum) and organic matter quality to predict the effects of anthropogenic nutrient loading to stream ecosystems.

Acknowledgments

We thank J.L. Tank and L. Taylor for helpful suggestions in designing the artificial substrata. We are grateful to M. Hidalgo, D. Vázquez and J.B. Logue for help with field work. We thank OTS and the staff at La Selva Biological Station for logistical support. This research was supported by NSF DEB-0075339, 0545463 to C.M. Pringle, F.J. Triska and A. Ramírez; NSF 0434503 and a UGA Dissertation Completion award to M. Ardón.

References

APHA (1998) *Standard Methods for the Examination of Water and Wastewater*, 20th edn. American Public Health Association, Washington, D.C., U.S.A.

Ardón M. (2006) Effects of Leaf Litter Quality on Decomposition Dynamics in Lowland Neotropical Streams. Ph.D. Thesis, University of Georgia, Athens, GA, U.S.A.

Ardón M., Stallcup L.A. & Pringle C.M. (2006) Does leaf quality mediate the stimulation of leaf breakdown by phosphorus in Neotropical streams? *Freshwater Biology*, **51**, 618–633.

Battin T.J., Kaplan L.A., Newbold J.D. & Hansen C.M.E. (2003) Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature*, **426**, 439–442.

Benfield E.F. (1996) Leaf breakdown in stream ecosystems. In: *Methods in Stream Ecology* (Eds F.R. Hauer & G.A. Lamberti), pp. 579–589. Academic Press, San Diego, CA, U.S.A.

Bernhardt E.S. & Likens G.E. (2002) Dissolved organic carbon enrichment alters nitrogen dynamics in a forest stream. *Ecology*, **83**, 1689–1700.

Canhoto C. & Graça M.A.S. (1999) Leaf barriers to fungal colonization and shredders (*Tipula lateralis*) consumption of decomposing *Eucalyptus globulus*. *Microbial Ecology*, **37**, 163–172.

Crocker M.T. & Meyer J.L. (1987) Interstitial dissolved organic carbon in sediments of a southern Appalachian headwater stream. *Journal of the North American Benthological Society*, **6**, 159–157.

Dent C.L. & Grimm N.B. (1999) Spatial heterogeneity of stream water nutrient concentrations over successional time. *Ecology*, **80**, 2283–2298.

Dent C.L., Grimm N.B. & Fisher S.G. (2001) Multiscale effects of surface-subsurface exchange on stream water nutrient concentrations. *Journal of the North American Benthological Society*, **20**, 162–181.

Grattan R.M. & Suberkropp K. (2001) Effects of nutrient enrichment on yellow poplar leaf decomposition and fungal activity in streams. *Journal of the North American Benthological Society*, **20**, 33–43.

Greenwood J.L., Rosemond A.D., Wallace J.B., Cross W.F. & Weyers H.S. (2006) Nutrients stimulate leaf breakdown rates and detritivore biomass: bottom-up effects via heterotrophic pathways. *Oecologia*, **151**, 637–649.

Gulis V. & Suberkropp K. (2003) Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshwater Biology*, **48**, 123–134.

Gulis V., Rosemond A.D., Suberkropp K., Weyers H.S. & Benstead J.P. (2004) Effects of nutrient enrichment

on the decomposition of wood and associated microbial activity in streams. *Freshwater Biology*, **49**, 1437–1447.

Hedin L.O. (1990) Factors controlling sediment community respiration in woodland stream ecosystems. *Oikos*, **57**, 94–105.

Koetsier P.I., McArthur J.V. & Leff L.G. (1997) Spatial and temporal response of stream bacteria to sources of dissolved organic carbon in a blackwater stream system. *Freshwater Biology*, **37**, 79–89.

Leff L.G. (2000) Longitudinal changes in microbial assemblages of the Ogeechee River. *Freshwater Biology*, **43**, 605–615.

McArthur J.V., Marzolf G.R. & Urban J.E. (1985) Response of bacteria isolated from a pristine praire stream to concentration and source of soluble organic carbon. *Applied and Environmental Microbiology*, **49**, 238–241.

McNamara C.J. & Leff L.G. (2004) Response of biofilm bacteria to dissolved organic matter from decomposing maples leaves. *Microbial Ecology*, **48**, 324–330.

Meyer J.L. (1994) The microbial loop in flowing waters. *Microbial Ecology*, **28**, 195–199.

Meyer J.L. & Tate C. (1983) The effects of watershed disturbance on dissolved organic carbon dynamics of a stream. *Ecology*, **64**, 33–44.

Meyer J.L., Edwards R.T. & Risley R. (1987) Bacterial growth on dissolved organic carbon from a blackwater river. *Microbial Ecology*, **13**, 13–29.

Mulholland P.J., Elwood J.W., Newbold J.D., Webster J.R., Ferren L.A. & Perkins R.E. (1984) Phosphorus uptake by decomposing leaf detritus: effect of microbial biomass and activity. *Verhandlungen der Internationale Vereinigung für Theoretische und Angewandte Limnologie*, **22**, 1899–1905.

Olapade O.A. & Leff L.G. (2005) Seasonal response of stream biofilm communities to dissolved organic matter and nutrient enrichments. *Applied and Environmental Microbiology*, **71**, 2278–2287.

Pringle C.M. (1987) Effects of water and substratum nutrient supplies on lotic periphyton growth – an integrated bioassay. *Canadian Journal of Fisheries and Aquatic Sciences*, **44**, 619–629.

Pringle C.M. (1990) Nutrient spatial heterogeneity: effects on community structure, physiognomy, and diversity of stream algae. *Ecology*, **71**, 905–920.

Pringle C.M. & Bowers J.A. (1984) An in situ substratum fertilization technique: diatom colonization on nutrient-enriched, sand substrate. *Canadian Journal of Fisheries and Aquatic Sciences*, **41**, 1247–1251.

Pringle C.M. & Triska F.J. (1991) Effects of geothermal groundwater on nutrient dynamics of a lowland Costa Rican stream. *Ecology*, **72**, 951–965.

Pringle C.M. & Triska F.J. (1996) Effects of nutrient enrichment on periphyton. In: *Methods in Stream Ecology* (Eds F.R. Hauer & G.A. Lamberti), pp. 607–624. Academic Press, San Diego, CA, U.S.A.

Pringle C.M., Paaby-Hansen P., Vaux P.D. & Goldman C.R. (1986) In situ nutrient assays of periphyton growth in a lowland Costa Rican stream. *Hydrobiologia*, **134**, 207–213.

Pringle C.M., Naiman R.J., Bretschko G., Karr J.R., Oswood M.W., Webster J.R., Welcomme R.L. & Winterbourn M.J. (1988) Patch dynamics in lotic systems – the stream as a mosaic. *Journal of the North American Benthological Society*, **7**, 503–524.

Pringle C.M., Rowe G.L., Triska F.J., Fernandez J.F. & West J. (1993) Landscape linkages between geothermal activity, solute composition and ecological response in streams draining Costa Rica's Atlantic slope. *Limnology and Oceanography*, **38**, 753–774.

Ramírez A. & Pringle C.M. (2006) Fast growth and turnover of chironomid assemblages in response to stream phosphorus levels in a tropical lowland landscape. *Limnology and Oceanography*, **51**, 189–196.

Ramírez A., Pringle C.M. & Molina L. (2003) Effects of stream phosphorus levels on microbial respiration. *Freshwater Biology*, **48**, 1–10.

Royer T.V. & Minshall G.W. (2001) Effects of nutrient enrichment and leaf quality on the breakdown of leaves in a hardwater stream. *Freshwater Biology*, **46**, 603–610.

Sanford R.L., Paaby P., Luvall J.C. & Phillips E. (1994) Climate, geomorphology, and aquatic systems. In: *La Selva: Ecology and Natural History of a Neotropical Rainforest* (Eds L.A. McDade, K.S. Bawa, H.A. Hespenhedie & G.S. Hartshorn), pp. 19–33. University of Chicago Press, Chicago, IL, U.S.A.

Sobczak W.V., Findlay S. & Dye S. (2003) Relationships between DOC bioavailability and nitrate removal in an upland stream: an experimental approach. *Biogeochemistry*, **62**, 309–327.

Stallcup L.A., Ardón M. & Pringle C.M. (2006) Does nitrogen become limiting under high-P conditions in detritus-based tropical streams? *Freshwater Biology*, **51**, 1515–1526.

Stelzer R.S., Heffernan J. & Likens G.E. (2003) The influence of dissolved nutrients and particulate organic matter quality on microbial respiration and biomass in a forest stream. *Freshwater Biology*, **48**, 1925–1937.

Suberkropp K. (1998) Effects of dissolved nutrients on two aquatic hyphomucetes growing on leaf litter. *Mycological Research*, **8**, 998–1002.

Suberkropp K. & Weyers H. (1996) Application of fungal and bacterial production methodologies to decompos-

ing leaves in streams. *Applied and Environmental Microbiology*, **62**, 1610–1615.

Tank J.L. & Dodds W.K. (2003) Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. *Freshwater Biology*, **48**, 1031–1049.

Tank J.L. & Webster J.R. (1998) Interaction of substrate and nutrient availability on wood biofilm processes in streams. *Ecology*, **79**, 2168–2179.

Tank J.L., Bernot M.J. & Rosi-Marshall E.J. (2006) Nitrogen limitation and uptake. In: *Methods in Stream Ecology*, 2nd edn. (Eds F.R. Hauer & G.A. Lamberti), pp. 213–238. Academic Press, Burlington, MA, U.S.A.

Webster J.R. & Benfield E.F. (1986) Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics*, **17**, 567–594.

Wilcox H.S., Wallace J.B., Meyer J.L. & Benstead J.P. (2005) Effects of labile carbon addition on a headwater stream food web. *Limnology and Oceanography*, **50**, 1300–1312.

(Manuscript accepted 26 April 2007)