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Nutrient limitation of epilithic and epixylic biofilms in 10 North American streams

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SUMMARY

1. Nutrient diffusing substrata were used to determine the effect of added inorganic nitrogen (N) and phosphorus (P) on the development of epilithic and epixylic biofilms in 10 North American streams. Four treatments of diffusing substrata were used: Control (agar only), N addition (0.5 M $NaNO_3$), P addition (0.5 M KH_2PO_4), and N + P combined (0.5 M $NaNO_3$ + 0.5 M KH_2PO_4). Agar surfaces were covered with glass fibre filters (for epilithon) or discs of untreated white oak wood veneer (for epixylon).
2. We found that if algae showed significant response to nutrient addition, N limitation (either N alone or N with P) was the most frequent response both on GF/F filters and on wood. Despite the low dissolved nutrient concentrations in our study streams, more than a third of the streams did not show any response to N or P addition. In fact, P was never the sole limiting nutrient for algal biofilms in this study.
3. Nutrient addition influenced algal colonisation of inorganic versus organic substrata in different ways. The presence of other biofilm constituents (e.g. fungi or bacteria) may influence whether algal biomass on wood increased in response to nutrient addition. Algae on organic and inorganic substrata responded similarly to nutrient addition in only one stream.
4. Fungal biomass on wood was nutrient limited in six of 10 study streams. N limitation of fungal biomass (with or without secondary P limitation) was most frequent, but P limitation did occur in two streams.
5. Our results show that biomass responses to nutrient addition by the heterotrophic and autotrophic components of the epixylic biofilm were different, though both experienced the same stream nutrient conditions. For algae and fungi growing on wood, limiting nutrients were rarely similar. Only three of nine streams showed the same biomass response to nutrient addition, including two that showed no significant change in biomass despite added nutrients.

Keywords: biofilm, epilithon, fungi, nitrogen, nutrients, periphyton, phosphorus, stream

Introduction

Microbial biofilms are largely responsible for stream processes such as primary production, community respiration, nutrient uptake and retention, and decomposition of particulate organic matter. While autotrophs (primarily algae) control the transfer of atmospheric carbon to higher trophic levels, microbial colonisation by bacteria and fungi is critical to the transfer of allochthonous particulate carbon through stream food webs. However, primary production and detrital decomposition are influenced by nutrient availability. In streams, dissolved nutrients are continuously delivered to colonised surfaces via unidirectional water flow. Nevertheless, nutrients

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(primarily nitrogen (N) and phosphorus (P)) are often limiting to algae, bacteria and fungi in these systems (e.g. Pringle *et al*., 1986; Tank & Webster, 1998; Francoeur *et al*., 1999; Wold & Hershey, 1999).

Research on nutrient limitation in streams has centred primarily on algae (i.e. periphyton or epilithon). Factors important to the accrual of autotrophic biomass include nutrients (Borchardt, 1996), light (Hill, 1996), temperature, invertebrate grazing (Steinman, 1996), and scouring caused by increased flow (Biggs & Close, 1989; Peterson, 1996). In general, however, there is a positive relationship between nutrient concentrations in the water column and benthic chlorophyll across many streams (Dodds, Smith & Zander, 1997). In contrast to the algal-dominated biofilms colonising inorganic substrata, fungi comprise the major biotic component of heterotrophic decomposers in streams dominated by allochthonous organic matter (Suberkropp & Klug, 1976). Fungi play a major role in the conditioning of woody debris and leaf litter, thereby making detritus a more easily assimilated food resource for consumers (Barlocher, 1985; Hax & Golladay, 1993). In fact, fungi may comprise up to 90% of the microbial biomass colonising large particulate organic matter in heterotrophic food webs (Aumen *et al*., 1983; Findlay & Arsuffi, 1989; Tank & Winterbourn, 1996). Heterotrophs, such as fungi, are not only influenced by environmental conditions such as temperature and dissolved nutrients (Suberkropp & Chauvet, 1995; Tank & Webster, 1998), but are also influenced by the quality of the substratum they colonise (e.g. lignin content, C : N ratio) (Peterson *et al*., 1993; Gessner & Chauvet, 1994). Given these differences between autotrophs and heterotrophs, we can ask (a) whether the same nutrients limit both algae and fungi growing in the same stream or on the same substratum and (b) whether the magnitude of biomass response to the addition of these nutrients is the same for both algae and fungi. Relatively few studies have examined the effect of water column nutrients on heterotrophic activity, despite their important role in stream ecosystem function. Moreover, we know of no simultaneous test for nutrient limitation of both autotrophs and heterotrophs. Because algae grow on both inorganic and organic substrata, we analysed the response of algae to nutrient addition on both substratum types.

We investigated nutrient (N and P) limitation in heterotrophic (fungal) and autotrophic (algal) biofilms using nutrient-diffusing substrata: (1) across streams in different biomes with different nutrient and light regimes and (2) on inorganic and organic substrata. We compared the biofilm response with nutrient addition in 10 first or second order streams in North America, yielding a 'snapshot' of N and P limitation for both algae and fungi. These sites range from closed-canopy, shaded streams to others with little riparian vegetation and open channels. Our intent was not to describe the geographical distribution of N and P limitation, but rather to explore the possible range of responses to N and P addition between fungi and autotrophic algae in streams. Differentiating between the response to nutrient addition of the algal and fungal components of biofilms is critical to understanding how nutrients may differentially influence or control stream ecosystem function through heterotrophic and/or autotrophic pathways.

Methods

Our 10 study streams were located in eight different biomes, ranging from tropical to tundra (Table 1). Seven of the 10 streams had closed canopies (i.e. >50% riparian shading) and heterotrophic processes strongly dominated stream metabolism (Mulholland *et al*., 2001; Table 2). The remaining three streams had open canopies and a higher rate of primary production (Tables 1 and 2). The streams varied somewhat in physical and chemical characteristics but were in general small (<200 L s^{-1} baseflow discharge) and oligotrophic (dissolved inorganic N (DIN) <60 μg L^{-1} and soluble reactive phosphorus (SRP) <15 μg L^{-1}, Table 2). The ratio of molar DIN : SRP ranged from 2 to 36 across the 10 sites (Table 2).

Nutrient diffusing substrata (NDS) were constructed using 60 mL plastic containers filled with a 2% (by weight) agar solution amended with 0.5 M KNO_3 (N treatment), 0.5 M NaH_2PO_4 (P treatment), both (N + P treatment), or not amended as a control (C treatment). We placed either Whatman (Kent, UK) GF/F glass fibre filters (0.7 μm retention) or 1-mm thick untreated white oak (*Quercus alba* G. Lumis) veneer discs (from heartwood; Constantines Wood Center, Ft. Lauderdale, FL, USA) across the tops of the containers to cover the agar completely and serve as either inorganic or organic substrata for biofilm colonisation. The wood veneer technique has been used successfully to assess nutrient limitation in

Table 1 Study sites where bioassays were conducted

Site (State)	Location	Description	Canopy	Season (month)	Incubation time (day)
Upper Ball Creek, North Carolina (NC)	35°N, 83°W	Temperate deciduous forest	Closed	Autumn (November)	42
Walker Branch, Tennessee (TN)	36°N, 84°W	Temperate deciduous forest	Closed	Spring (March)	21
Sycamore Creek, Arizona (AZ)	33°N, 112°W	Sonoran desert	Open	Spring (May)	22
Bear Brook, New Hampshire (NH)	44°N, 72°W	Temperate deciduous forest	Closed	Summer (June)	21
Gallina Creek, New Mexico (NM)	36°N, 106°W	High desert montane forest	Closed	Autumn (September)	21
Quedebra Bisley, Puerto Rico (PR)	18°N, 66°W	Tropical forest	Closed	Winter (February)	21
Kings Creek, Kansas (KS)	39°N, 94°W	Prairie	Open	Spring (April)	19
Eagle Creek, Michigan (MI)	42°N, 85°W	Temperate, low gradient forest	Closed	Summer (June)	21
Mack Creek, Oregon (OR)	44°N, 122°W	Temperate rainforest	Closed	Summer (July)	21
E 1, Alaska (AK)	68°N, 149°W	Tundra	Open	Summer (July)	21

epixylon (Tank & Winterbourn, 1995, 1996; Tank & Webster, 1998). This represents a modification of bioassay techniques developed previously by Winterbourn (1990) and Corkum (1996).

Five replicates of each nutrient treatment for both organic and inorganic substrata were placed on the stream bottom in plastic racks for 21 ± 2 days, except in Upper Ball Creek where low winter stream temperature resulted in very slow colonisation times and incubation was for 42 days (Table 1). Laboratory assays have previously shown that the rate of nutrient diffusion from the 2% agar cups was constant through 17 days and then declined only slightly until day 21 (J.L. Tank, unpublished data). At the end of the incubation period, filters were removed and frozen until analysis for chlorophyll *a* (to estimate algal biomass). Wood veneer discs were removed, split, and half of each disc was frozen for later analysis of chlorophyll *a* and the other half immediately fixed with 5 mL of high-performance liquid chromatography (HPLC)-grade methanol for later extraction of ergosterol (an indicator of fungal biomass). Within 48 h of collection, chlorophyll *a* extracts were analysed spectrophotometrically or fluorometrically with correction for phaeophytin by standard methods (APHA, 1995). Ergosterol was extracted and analysed using an HPLC as reported previously (Newell, Arsuffi & Fallon, 1988; Tank & Webster, 1998) and expressed as fungal biomass using a general conversion factor of 6 mg ergosterol g^{-1} fungal biomass (Newell *et al*., 1988).

To determine whether algal or fungal biomass on our artificial substrata (GF/F filters or wood veneer) approximated *in situ* biomass on natural substrata (stream rocks and submerged woody debris) in our 10 study streams, we estimated epilithic algal biomass (as chlorophyll *a*) and fungal biomass on wood (as ergosterol) just prior to the implementation of the NDS and compared biomass estimates from natural substrata to those from the control (non-nutrient) treatments of the NDS. *In situ* epilithic biomass was determined at each site using 12 randomly selected rock scrapings of known area and filtering the epilithon slurry from each rock onto ashed GF/F filters and then extracting for chlorophyll *a* as described above for the NDS. Fungal biomass in the epixylon was obtained by scraping a known area of submerged wood and filtering the epixylic slurry in a fashion similar to the epilithon filtering ($n = 12$ per site). The resulting filter was then extracted for ergosterol as described above. Both *in situ* chlorophyll *a* and fungal biomass were then expressed per unit surface area of scraping.

Stream water nutrient analyses were conducted on water samples taken weekly during the NDS incubations. All water samples were immediately filtered

Table 2 Physical and chemical characterisation of 10 study sites as abbreviated by state (see Table 1). Data reported are weekly averages over the study period, except for daily light and metabolism which represent one sampling date at the beginning of the incubation period

	Physical			Water chemistry							Metabolism				
Site	Q ($L\ s^{-1}$)	Depth (m)	Daily light ($mol\ m^{-2}\ d^{-1}$)	Water velocity ($cm\ s^{-1}$)	Water temp. (°C)	NH_4^+ ($\mu gN\ L^{-1}$)	NO_3^- ($\mu gN\ L^{-1}$)	DON ($\mu gN\ L^{-1}$)	SRP ($\mu gP\ L^{-1}$)	DIN : SRP (Molar)	Epilithon ($gAFDM\ m^{-2}$)	Epilithon ($mg\ Chl\ m^{-2}$)	GPP ($gO_2\ m^{-2}\ d^{-1}$)	CR	P : R ratio
NC	130	0.18	0.8	10.6	7.2	3.3	2.3	20.3	2.9	4.3	1.26	0.59	0.06	32.3	0.002
TN	18	0.05	15.0	6.8	12.4	4.1	18.7	–	3.3	15.3	3.8	12.0	1.1	6.3	0.18
AZ	43	0.04	51.0	28.6	23.0	6.0	9.0	179.0	14.0	2.4	18.0	53.0	15.0	8.3	1.8
NH	9	0.09	2.2	1.9	14.3	4.3	54.4	153.0	3.6	36.1	2.66	7.8	0.2	9.1	0.02
NM	4	0.03	6.8	9.9	7.2	4.7	4.2	127.0	8.0	2.5	3.49	8.65	0.4	6.7	0.06
PR	20	0.13	0.3	3.0	22.0	3.3	167.0	119.0	14.3	26.4	3.54	9.8	0.1	9.0	0.01
KS	16	0.15	38.0	11.3	15.5	3.0	2.3	–	3.3	3.6	76.0	63.0	1.8	2.4	0.75
MI	202	0.18	18.0	23.6	23.0	12.0	17.5	229.0	3.1	21.1	5.84	7.4	1.2	6.4	0.19
OR	57	0.16	3.8	7.6	13.1	2.2	59.2	50.0	13.0	10.5	2.9	13.0	1.9	11.2	0.17
AK	134	0.10	–	13.0	9.8	2.8	13.6	150.0	1.8	20.2	–	5.8	1.1	0.3	3.9

through Gelman (Pall Corporation, Port Washington, NY, USA) A/E glass fibre filters (0.45 μm pore retention) in the field and then frozen for later chemical analysis. Water was analysed for ammonium, nitrate + nitrite, soluble reactive P, and total dissolved N using standard colorimetric methods (APHA, 1995; Table 2).

A two-factor analysis of variance (ANOVA) was used to test whether algal or fungal biofilms were significantly affected by N enrichment (presence or absence of $NaNO_3$ in agar) or P enrichment (presence or absence of KH_2PO_4 in agar) (Dube, Culp & Scrimgeour, 1997). *Post-hoc* least-squares means (LSM) followed significant ANOVA ($P < 0.05$) to differentiate between biomass means. If the biomass data were not normally distributed or the variances were unequal, the data were log-transformed prior to analysis. Possible outcomes from the ANOVA on the bioassays are summarised in Table 3. Single nutrient limitation was indicated when just one of the additions (N or P) elicited a positive response, but the interaction term in the ANOVA was not significant. If neither N nor P alone significantly increased biomass ($P > 0.05$), but N and P added together (N + P) did (i.e. the interaction term in the ANOVA was significant, $P < 0.05$), we considered the biofilm to be colimited by both N and P. Similarly, there could also be colimitation by both N and P if, when added separately, they each stimulated biomass relative to controls, but the positive N and P responses were not different from each other. Secondary limitation was indicated if N or P alone significantly increased biomass, both N and P added together caused an even greater increase in biomass, and the interaction term for the ANOVA was significant.

Table 3 Interpretation of responses to N and P addition. A diamond in N or P treatment indicates a significant N or P effect in the two-way ANOVA ($P < 0.05$) and a diamond in the NXP treatment indicates a significant interaction between the two treatments

Interpretation	N effect	P effect	Interaction NXP
N limited	◆		
P limited		◆	
N and P colimited			◆
N and P colimited	◆	◆	
N and P colimited	◆	◆	◆
1°N limited, 2°P limited	◆		◆
1°P limited, 2°N limited		◆	◆
Not limited by N or P			

Whether a particular nutrient is found to be limiting or not is partially a function of the statistical power of the experiment (Francoeur, 2001). For this reason, we found it useful to compare in addition the relative magnitude of the response of algal or fungal biomass to nutrient addition between streams and substrata types. We modified the approach of Brett & Goldman (1997) and the treatment response data were transformed by calculating the logarithmic ratio of the treatment (N, P, or N + P) relative to the control. For example, for the addition of N, the N biomass response would be log (nitrogen mean/control mean). In this way, the response variables (either chlorophyll *a* or fungal biomass) were normalised across streams by scaling the mean treatment response (to N, P, or N + P) relative to the control treatment (N : C, P : C, N + P : C); the higher the ratio, the greater the response to nutrient addition. We used ANOVA to compare the biomass responses to nutrient addition among substratum types (chorophyll *a* on GF/F versus wood veneers) and between response variables on the same substratum type (fungal biomass versus chlorophyll *a* on wood). *Post-hoc* LSM followed significant ANOVA ($P < 0.05$) to differentiate between biomass responses. Linear regression was used to test for significant relationships between response to nutrient addition (as log relative response) and the independent variables described in Table 2, such as stream water nutrient concentrations or light.

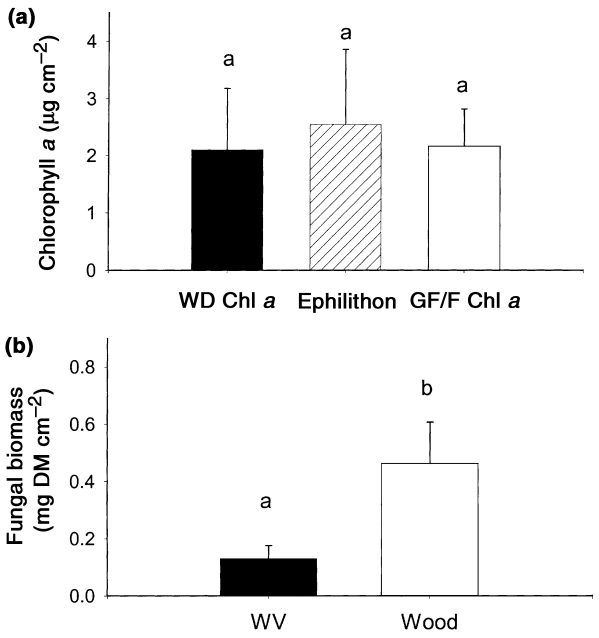

Fig. 1 (a) Mean chlorophyll *a* from the control treatment at all sites using wood veneer substrata (WD chl *a*), natural epilithon colonising stream rocks (epilithon), and control treatment using GF/F filters (GF/F chl *a*). (b) Mean fungal biomass from the control treatment at all sites using wood veneer substrata (WV) and *in situ* small woody debris (wood). Means ± standard errors (SE) are plotted and $N = 5$ for each bar. Letters refer to comparisons of treatments among all streams and bars with same letters were not significantly different (ANOVA, $P > 0.05$).

Results

Are the biofilms on the GF/F filters and wood veneers representative of in situ *biofilms?*

Chlorophyll *a* concentration on both wood veneers and GF/F filters was not significantly different from ambient chlorophyll *a* on stream rocks (ANOVA, $P > 0.05$, Fig. 1a), so the 3-week incubation time resulted in a representative epilithic biomass on artificial substrata. In contrast, fungal biomass on wood veneer was significantly lower than on surfaces of *in situ* small woody debris (ANOVA, $P = 0.04$), so the 3-week incubation time resulted in lower fungal colonisation (Fig. 1b), consistent with previous work using veneers (Tank & Webster, 1998). We did not assess the community structure of biofilms, so we do not know if *in situ* biofilms are structurally similar to those that colonised our artificial substrata.

Nutrient limitation in algae

Chlorophyll *a* concentrations were similar between GF/F filters and wood veneers (Figs 2 and 3). On GF/F, chlorophyll *a* ranged from a minimum of 0.1 μg cm^{-2} for the P treatment in Puerto Rico to 13.2 μg cm^{-2} for the N + P treatment in Kansas (Fig. 2). On wood veneers, chlorophyll *a* ranged from 0.07 μg cm^{-2} for the N + P treatment in Puerto Rico to 19.3 μg cm^{-2} for the N + P treatment in Arizona (Fig. 3). Substratum type did not influence algal biomass for either controls or nutrient treatments and mean chlorophyll *a* concentrations on the substrata for each individual treatment were not different between wood and GF/F (paired *t*-test, for control, $P = 0.226$, N addition, $P = 0.793$, P addition, $P = 0.432$, and N + P addition, $P = 0.987$).

We tested for a significant algal response to nutrient addition using two-way ANOVA (Table 4). In about half of the streams, algal biomass was not

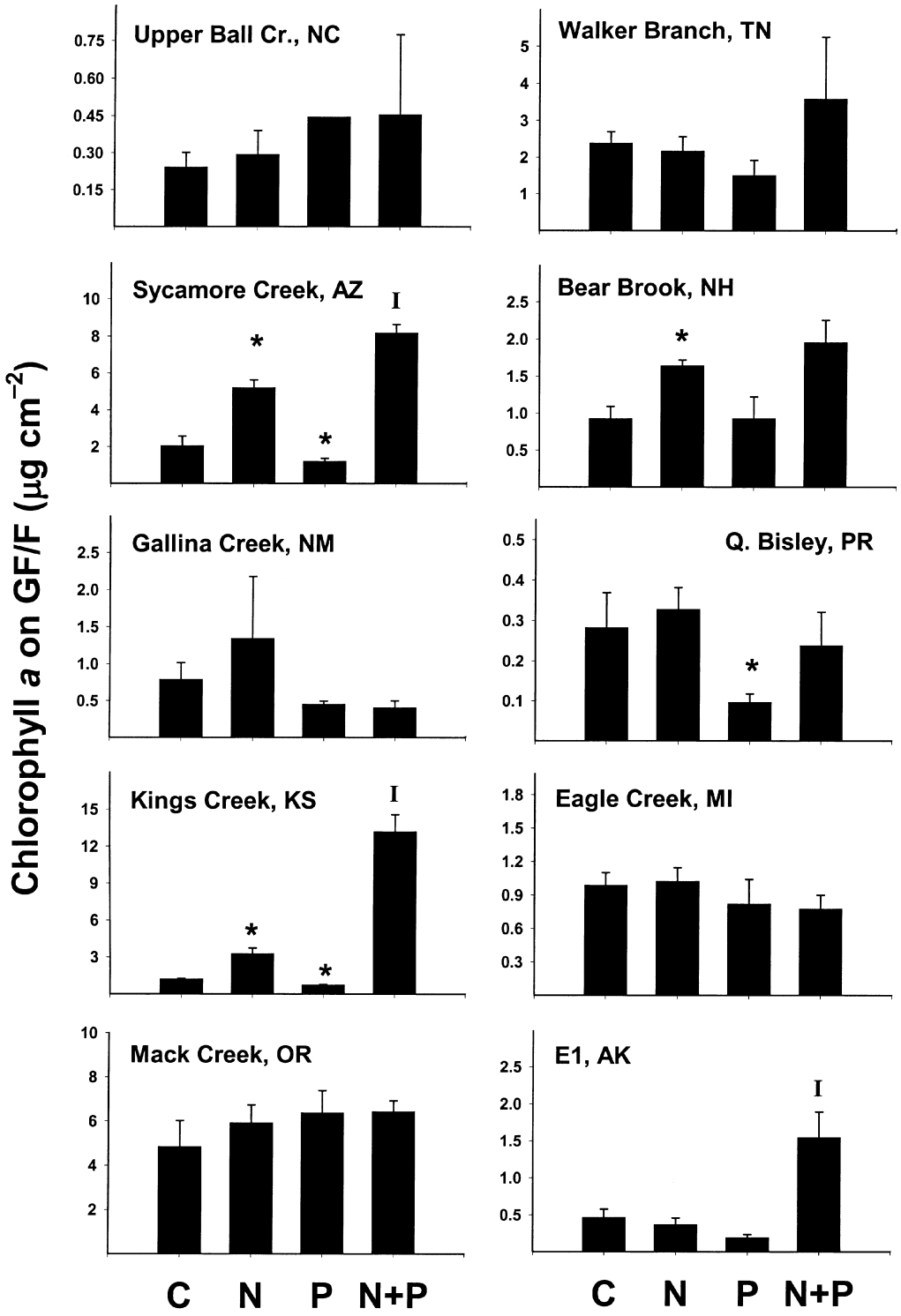

Fig. 2 Chlorophyll *a* on GF/F filters from the four nutrient treatments; control (C), N alone (N), P alone (P) and N and P added together (N + P) for each stream. Means ± standard errors (SE) are plotted and each bar represents $N = 5$ for each treatment at each site. Asterisks (*) above bars indicate a significant N effect, P effect, and (I) signifies a significant interaction term as determined by ANOVA ($P < 0.05$).

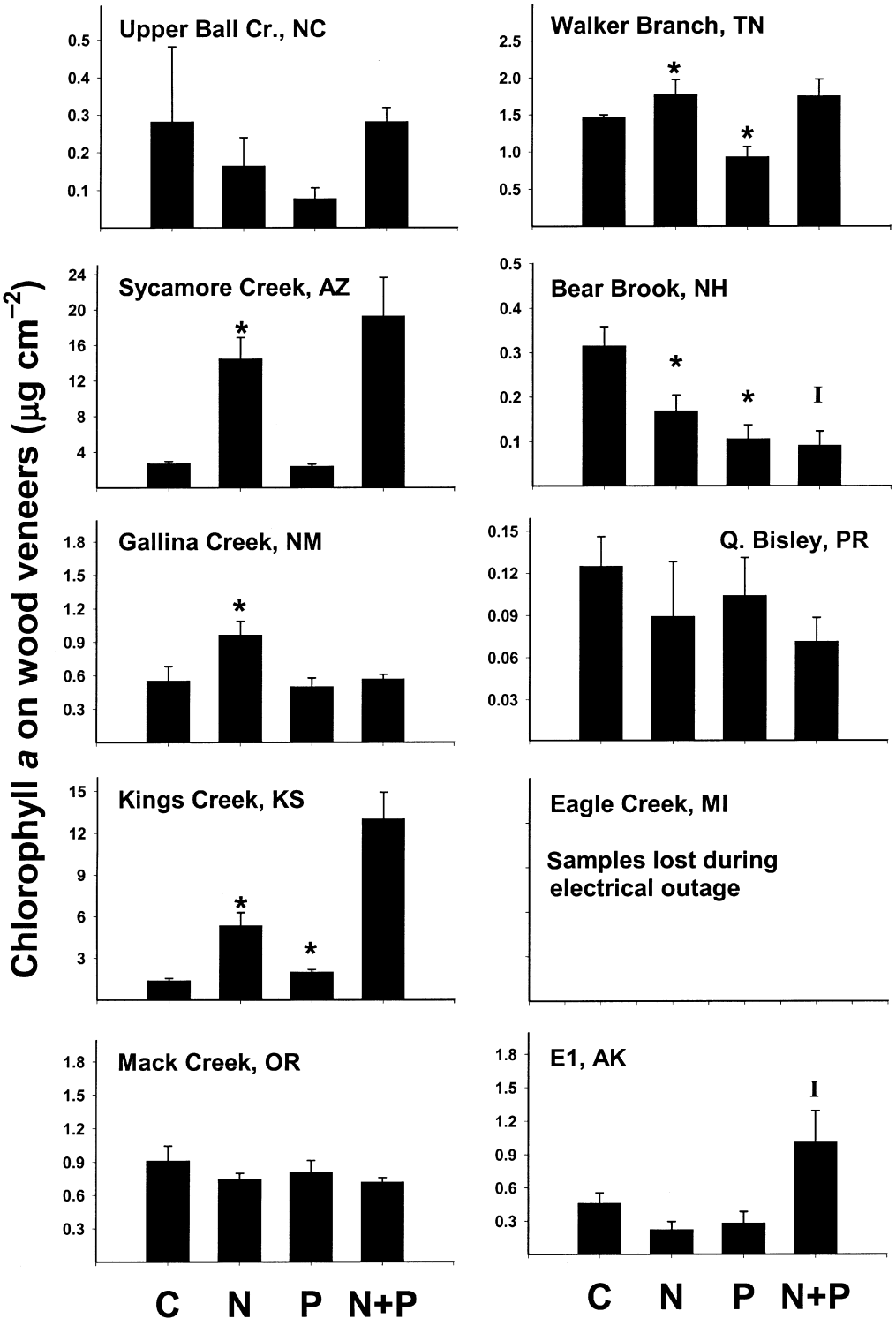

Fig. 3 Chlorophyll *a* on wood veneer substrata from the four nutrient treatments; control (C), N alone (N), P alone (P) and N and P added together (N + P) for each stream. Means ± standard errors (SE) are plotted and each bar represents $N = 5$ for each treatment at each site. Asterisks (*) above bars indicate a significant N effect, P effect, and (I) signifies a significant interaction term as determined by ANOVA ($P < 0.05$).

Table 4 Nutrient limitation status using ANOVA at each of the 10 study sites as abbreviated by state. Data for algae on wood in the Michigan stream were lost as a result of a power outage (Na). Relevant statistics from ANOVA are given only for the statistically significant positive responses to nutrient addition. For all analyses, d.f. = 1

Site	Algae on GF/F	Algae on wood	Fungi on wood
NC	–	–	*P* $F(p) = 25.58$, $P(p) < 0.0001$
TN	–	N $F(n) = 11.42$, $P(n) = 0.003$	N, 2P $F(n) = 12.72$, $P(n) = 0.002$ $F(p) = 5.91$, $P(p) = 0.025$ $F(np) = 7.84$, $P(np) = 0.011$
AZ	N, 2P $F(n) = 159.33$, $P(n) < 0.0001$ $F(np) = 22.43$, $P(np) < 0.0001$	N $F(n) = 90.6$, $P(n) < 0.0001$	N $F(n) = 65.03$, $P(n) < 0.0001$
NH	N $F(n) = 14.93$, $P(n) = 0.001$	–	N $F(n) = 29.17$, $P(n) < 0.0001$
NM	–	N $F(n) = 6.14$, $P(n) < 0.023$	N, 2P $F(n) = 27.6$, $P(n) < 0.0001$ $F(p) = 3.17$, $P(p) = 0.09$ $F(np) = 10.02$, $P(np) = 0.005$
PR	–	–	–
KS	N, 2P $F(n) = 353.28$, $P(n) < 0.0001$ $F(np) = 89.61$, $P(np) < 0.0001$	NP $F(n) = 120.85$, $P(n) < 0.0001$ $F(p) = 21.04$, $P(p) < 0.0001$	–
MI	–	Na	P $F(p) = 10.95$, $P(p) = 0.004$
OR	–	–	–
AK	NP $F(np) = 13.02$, $P(np) = 0.002$	NP $F(np) = 9.02$, $P(np) = 0.007$	–

N: N limitation alone, P: P limitation alone, –: no significant effect of nutrient addition, NXP: colimitation of N and P, and N, 2P: N limitation with 2°P limitation, F(n): Fstat for the N treatment, *P*(n): *P*-value for N treatment, *F*(p): Fstat for the P treatment, *P*(p): *P*-value for P treatment, *F*(np): Fstat for the NP treatment, *P*(np): *P*-value for NP treatment.

nutrient limited; six streams on the inorganic GF/F filters (NC, TN, NM, PR, MI, OR) and four streams on wood (NC, NH, PR, OR). Algal biomass on filters was limited by N alone in only a single stream (NH), but in three streams on wood veneers (AZ, NM, TN). Nitrogen limitation with secondary P limitation was more common for algae on filters (AZ, KS) but did not occur on wood. Co-limitation by both N and P on filters occurred only in the Alaskan stream, but was found in two streams on veneers (KS, AK). We saw no significant positive response of algae to P addition on either substratum in any of the 10 streams. Phosphorus addition suppressed algal biomass in three streams on filters (AZ, KS, PR) and in two streams on veneers (TN, NH). Autotrophs responded similarly on organic and inorganic substrata in only four of 10 streams, three showing no significant nutrient limitation (NC, PR, OR) and, when there was a statistically significant response, only the Alaskan stream showed the same treatment effect (Table 4).

Heterotrophic response to nutrient addition

The lowest estimates of fungal biomass occurred at the New Hampshire site (0.01 mg dry mass (DM) cm^{-2} for both control and P treatments), whereas fungal biomass was highest in Tennessee, both for control (0.25 mg DM cm^{-2}) and nutrient treatments (0.79 mg DM cm^{-2}, N + P) (Fig. 4). As in the autotrophs, there was no significant fungal response to nutrient addition in four of our study streams (KS, OR, PR, AK) (Table 4). Fungal biomass was limited by N alone in two streams (AZ, NH) and by P alone in two streams (NC, MI) (Table 4). Nitrogen limitation of fungal biomass with secondary P limitation also occurred in two streams (NM, TN), but there were no cases of colimitation by N and P.

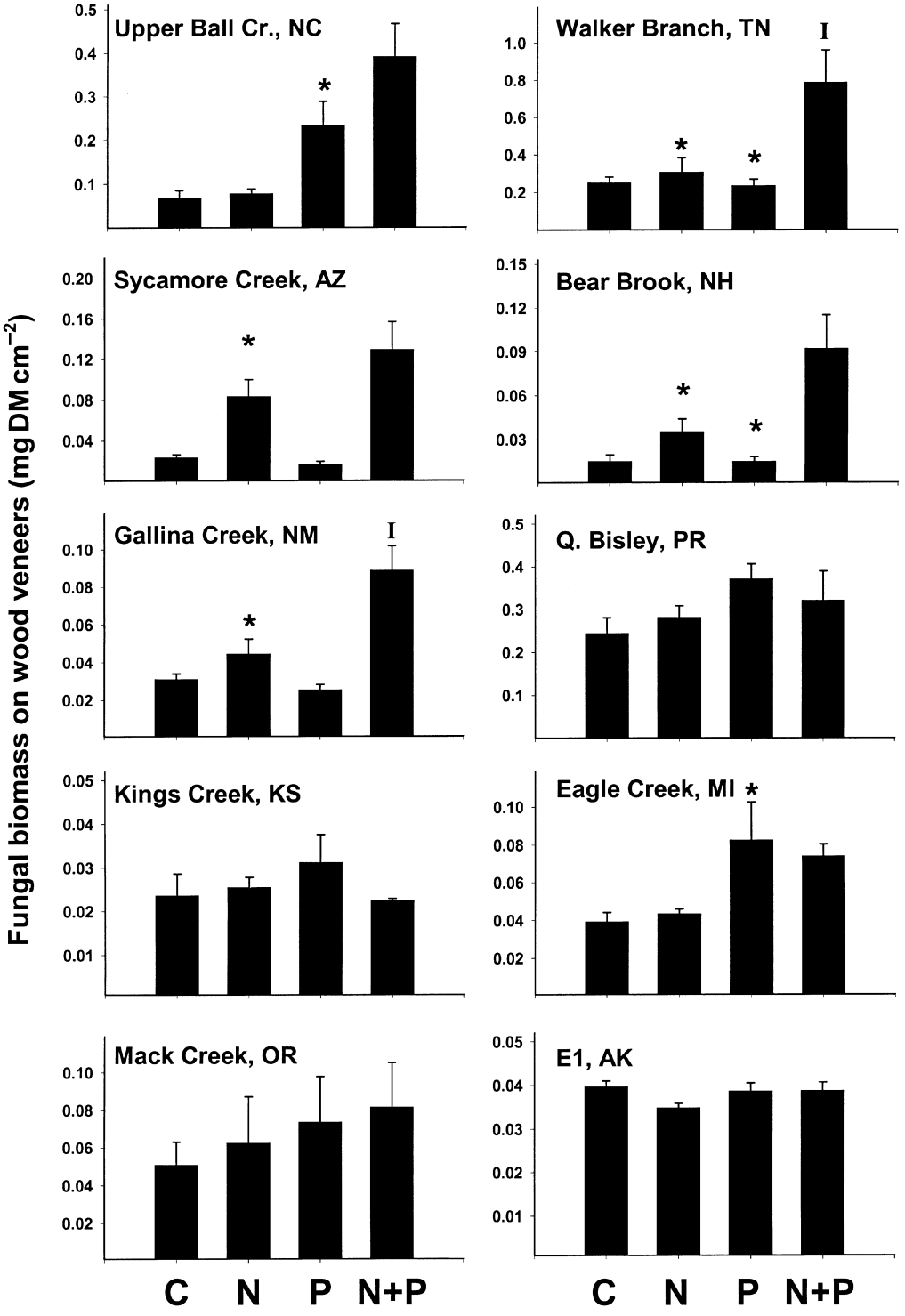

Fig. 4 Fungal biomass on wood veneer substrata from the four nutrient treatments; control (C), N alone (N), P alone (P) and N and P added together (N + P) for each stream. Means ± standard errors (SE) are plotted and each bar represents $N = 5$ for each treatment at each site. Asterisks (*) above bars indicate a significant N effect, P effect, and (I) signifies a significant interaction term as determined by ANOVA ($P < 0.05$).

Lower biomass after P addition occurred at only one site (TN).

Comparing the magnitude of the biomass response to nutrient addition

For algae on GF/F filters, there was no significant difference between the biomass responses for the N and N + P treatments, but both were higher than the response to P addition (ANOVA, $F = 5.56$, $P = 0.009$ followed by LSM, Fig. 5). For algae on wood, there was more variability among streams and there were no significant differences in the mean biomass response to nutrient treatments (ANOVA, $F = 2.53$, $P = 0.101$, Fig. 5). Analysis of pooled chlorophyll *a* data showed no significant influence of substratum type on the magnitude of biomass response by autotrophs to nutrient enrichment for the three treatments (N, P, and N + P) (Two-way ANOVA, $P = 0.211$). As NDS bioassays were conducted during different seasons (Table 1), we examined the biomass response data for chlorophyll *a* by season and found that there was a seasonal component (ANOVA, $F = 46.73$, $P < 0.0001$) and that bioassays conducted during spring had the greatest response, followed by summer, and then the winter and autumn experiments. The high biomass response by chlorophyll *a* in spring was influenced by the bioassay response in two (of three in total) high-light streams conducted at that time (AZ, KS). When the open-canopy streams (AZ, KS, AK) were analysed separately from shaded, the high-light streams generally had a higher biomass response (ANOVA, $F = 76.60$, $P < 0.0001$).

There was no seasonal pattern in fungal biomass response on wood. For fungi, the biomass response of the N and the P treatments was significantly lower than for N + P addition (ANOVA, $F = 4.01$, $P = 0.03$, followed by LSM, Fig. 5). The geometric mean fungal biomass increased by 26 to 43% for the N and P treatments, respectively, but there was a 157% increase as a result of N + P addition. On wood, the response to nutrient addition was different between autotrophic (i.e. algal chl *a*) and heterotrophic (i.e. fungal biomass) portions of the epixylic biofilm (ANOVA, $F = 6.85$, $P = 0.012$, followed by LSM), resulting primarily from a difference in response to P addition (Fig. 5). Looking at the responses to nutrient addition stream by stream, the consistency of nutrient limitation for autotrophs versus heterotrophs on wood veneers was low and only three streams showed the same nutrient response on both substrata (N limitation in AZ, no response in PR and OR) (Table 4).

Can the biomass response to nutrient addition be correlated with physiochemical variables?

For each nutrient treatment, the biomass response of algae on GF/F filters and wood, as well as that of fungi on wood, were related to a number of physiochemical stream variables, including stream water

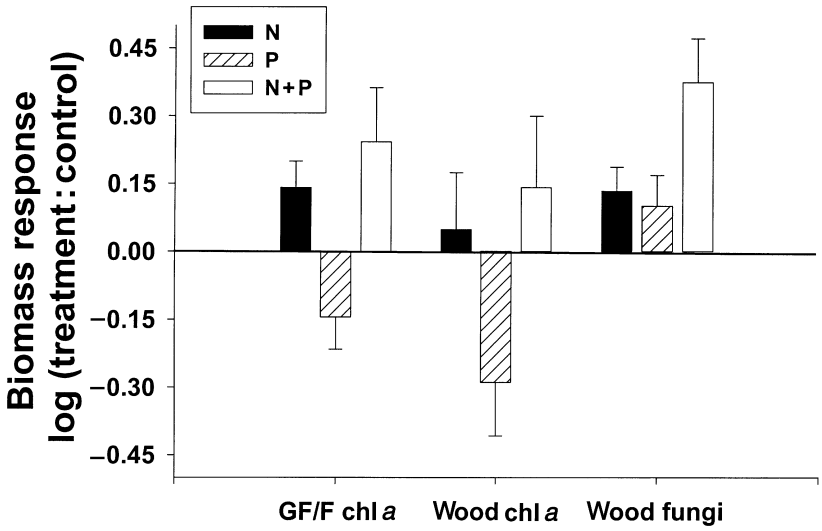

Fig. 5 Mean biomass response to nutrient addition (either chlorophyll *a* or fungal biomass) plotted by treatment and substratum type where biomass response is the log ratio of the treatment divided by the unenriched controls [i.e. nitrogen = log (nitrogen/control)]. Means ± standard errors (SE) are plotted for chlorophyll *a* on filters (GF/F chl *a*, $N = 10$ streams), chlorophyll *a* on wood veneers (wood chl *a*, $N = 9$ streams), and fungal biomass on wood (wood fungi, $N = 10$ streams).

chemistry, light [as photosynthetically active radiation (PAR)], whole-stream metabolism, stream temperature and depth (See Table 2). For algae, if there was a significant response to nutrient addition, N limitation (alone or in conjunction with P limitation) was most frequent and chlorophyll *a* concentrations were most strongly influenced by water chemistry and light (as PAR). On GF/F filters, chlorophyll *a* biomass response to N addition was inversely related to water column molar DIN : SRP ratios ($r^2 = 0.52$, $P = 0.02$, Fig. 6), but this relationship was only marginally significant on wood ($r^2 = 0.40$, $P = 0.06$, data not shown). In contrast, we found a positive correlation between light and the algal response to N addition on both filters ($r^2 = 0.44$, $P = 0.05$) and wood ($r^2 = 0.86$, $P = 0.001$) (Figs 7a,b), but this relationship was strongly influenced by our two high-light streams; a prairie stream in Kansas and a desert stream in Arizona. Similarly, autotrophic response to N + P addition was also influenced by light on both substrata (for GF/F, $r^2 = 0.44$, $P = 0.05$, and wood, $r^2 = 0.87$, $P = 0.001$).

Only the fungi showed significant P limitation. However, the fungal biomass response to P addition was not related to light or water chemistry, but rather was positively correlated with stream depth ($r^2 = 0.78$, $P = 0.001$) (Fig. 8). In this case, deeper streams, which may have lower light penetration and a relatively smaller autotrophic component in the epixylon, had greater response to P addition. This result is robust given the wide range of streams we studied.

Discussion

Autotrophic response to nutrient addition

Many studies have shown that autochthonous production is affected by nutrients (e.g. Bott, 1983; Grimm & Fisher, 1986; Pringle, 1987; Meyer *et al.*, 1988; Hart & Robinson, 1990; Peterson *et al.*, 1993; Rosemond, 1993; Rosemond *et al.*, 1993). In this study, we have used NDS bioassays of chlorophyll *a*, as an indicator of algal biomass, to assess potential nutrient limitation in streams in various biomes. In the 10 streams used in this study, DIN ranged from 5 to 170 μg L^{-1}, and SRP ranged from 2 to 14 μg L^{-1} (Table 2). These streams would be considered by most to be 'low nutrient' streams (especially for P) and therefore we predicted that biofilms would be limited in some way by nutrient availability. We recognise that biomass does not equal productivity *per se*, and our estimates only provide a snapshot in time with regard to nutrient limitation (Francoeur, 2001).

When nutrients were limiting, N limitation (either alone or with secondary limitation by P) was the most frequent response on both inorganic and

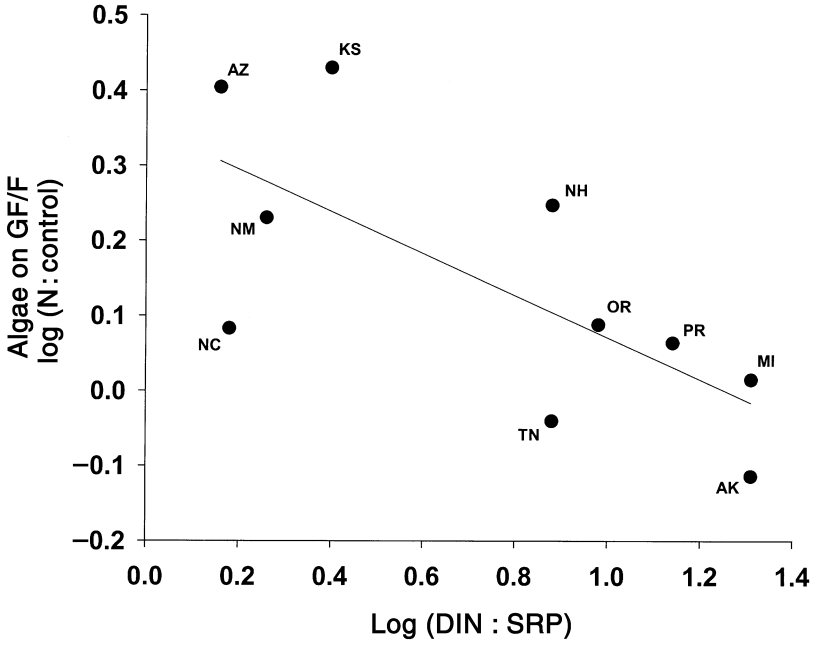

Fig. 6 Algal biomass response (as chlorophyll *a*) on GF/F filters to added nitrogen plotted against the log molar ratio of water column dissolved inorganic nitrogen (DIN) to soluble reactive phosphorus (SRP) concentrations. There was a significant negative relationship between algal N response versus DIN : SRP ($r^2 = 0.52$, $P = 0.02$).

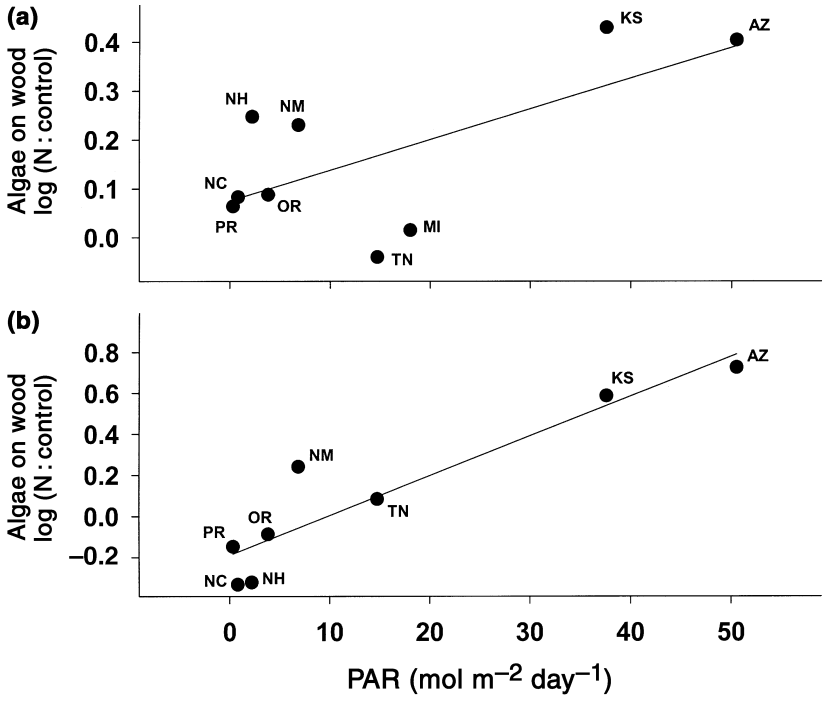

Fig. 7 Biomass response to N addition for algae (as chlorophyll *a*) on GF/F filters (a) and wood veneers (b) plotted against photosynthetically active radiation (PAR). There was a significant positive relationship between algal N response versus PAR (on GF/F, $r^2 = 0.44$, $P = 0.05$, on wood, $r^2 = 0.86$, $P = 0.001$).

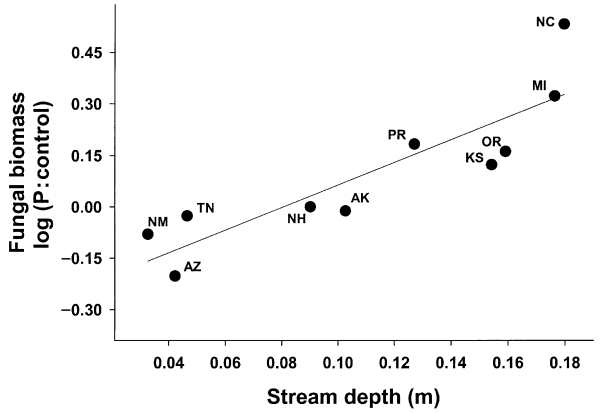

Fig. 8 Fungal biomass response on wood veneer (estimated using ergosterol concentrations) to added phosphorus plotted against average stream depth (m). There is a significant positive relationship between fungal response to P addition versus depth ($r^2 = 0.78$, $P = 0.001$).

organic substrata but, considering the low nutrient concentrations in our study streams, we were surprised that over one third of the streams were not nutrient limited (Table 4). We recognise that this may be a result of low statistical power (Scrimgeour & Chambers, 1997; Francoeur, 2001), but, it could also indicate that biofilm nutrient demand is being met, despite low water column nutrient concentration, or that there is another limiting factor (e.g. light availability, invertebrate grazing, scouring by floods). Low light, rather than nutrients, controlled algal biomass in a low-nutrient forested stream within the same basin as our NC site where Lowe, Golladay & Webster (1986) found no response by algae in a similar NDS experiment. In our study, the streams that showed the strongest response to added nutrients were the three open-canopy, high-light streams and, in general, the algal biomass response to added N increased with higher light levels on both substratum types (Fig. 7).

Grazing pressure by invertebrates may also affect the outcome of NDS bioassays (Lohman, Jones & Baysinger-Daniel, 1991; Dube *et al.*, 1997). Grazers may simply consume growth that is stimulated by nutrients in some systems. In this study, our goal was to assess the potential for nutrient limitation under *in situ* conditions, and for this reason we did not attempt to exclude grazers from the substrata. Nevertheless, in observing the deployed diffusing substrata, it was generally unusual to find invertebrates on bioassay surfaces during periodic checks, or at the time of retrieval, with the exception of snails on the substrata in Sycamore Creek AZ. Furthermore, if nutrient-enriched biofilms attracted grazers, we would have expected to see lower biomass on all

nutrient-enriched substrata, but we only saw reductions in biomass in some P treatments and not the corresponding N + P treatments. We never observed such an effect in the N treatments, nevertheless, we still acknowledge that grazing could have 'masked' responses to nutrient addition. In this case, the algal and fungal biomass we measured here are a net result of both biomass growth and loss.

Stream ecosystems are often not at equilibrium. Variations in discharge, groundwater inputs and precipitation can alter nutrient inputs and water column nutrient concentrations. An arctic tundra stream exhibited alternately P or N limited conditions depending on nitrate concentrations, which were influenced by stream discharge (Hullar & Vestal, 1989). Additionally, nutrient availability may change with depth into the biofilm. Although nutrients within biofilms are not often measured, flow has been shown to be highly attenuated in periphyton mats (Dodds, 1991), which may create a gradient of nutrient availability within biofilms. Spatial and temporal variation in nutrient limitation has been documented for streams in Minnesota (Wold & Hershey, 1999), Tennessee (Elwood *et al*., 1981) and New Zealand (Francoeur *et al*., 1999). We recognise that results from NDS studies must be interpreted cautiously, because they represent only a snapshot of stream nutrient conditions and, once streams have moved away from those conditions, nutrient limitation and response may differ greatly (Francoeur *et al*., 1999; Francoeur, 2001). Because we conducted bioassays in a wide variety of streams at different seasons, we assume that our results characterise a range of possible responses to nutrient enrichment rather than allowing characterisation of any individual stream or biome.

Despite very low SRP concentrations, often near our limits of detection (2–14 μg P L^{-1}), P was never the sole limiting nutrient for algal biofilms in this study. In fact, lower algal biomass as a result of P addition occurred three times on GF/Fs and once on wood. Previous NDS studies did not highlight this decline in biomass with P addition, but careful inspection of published data reveals some possible cases where it has occurred in other studies (e.g. Francoeur *et al*., 1999; Wold & Hershey, 1999; Francoeur, 2001). Perhaps such a response is reported less often than it really occurs. A *post hoc* diffusion study in the laboratory showed somewhat elevated SRP concentrations (<50 μg P L^{-1}) in water in which NDS had been placed. However, we have no way of knowing the P concentration at the surface of diffusing substrata at the time of incubation. Thus, we have no field data to support an explanation of the inhibitory response. Study of such a response could be a fruitful avenue for future research.

Effect of substratum type on autotrophic response

The response by algae to nutrient enrichment was not influenced by substratum type in our short-term studies. Chlorophyll *a* concentration and the magnitude of the biomass response were similar on both organic and inorganic substrata (Fig. 5). In cases where algae were nutrient limited, however, only the Alaskan stream showed similar limitation on both substrata. The presence of fungi could influence overall nutrient response by algae on wood. In general, biofilms on wood differ from inorganic substrata in that wood biofilms are dominated by fungal hyphae and may contain diatoms, while stone surfaces have no fungal hyphae (Tank & Winterbourn, 1996). Comparing biofilm development on organic (wood, leaves) and inorganic (stones) substrata in a New Zealand mountain stream, Tank & Winterbourn (1996) found that chlorophyll *a* concentration on wood could be as high as on stones, but endocellulase activity, an indicator of cellulose decomposition by heterotrophs, was present only on wood.

Nutrient limitation on organic substrata

Compared with algae, far fewer studies have explored patterns of nutrient limitation of heterotrophic colonisers. Fungi and bacteria colonising organic substrata can satisfy some of their N and P demand from the substratum, which may change dynamics of N and P limitation on organic substrata and make limitation patterns in wood biofilms distinct from epilithic biofilms. But when substratum nutrient concentration is insufficient, fungi can also obtain nutrients from the water column (e.g. Elwood *et al*., 1981; Mulholland *et al*., 1984). Previous work has focused primarily on the effect of nutrients on the decomposition of leaf litter. Like epilithon, both N and P have been shown to limit leaf decomposition

(Kaushik & Hynes, 1971; Howarth & Fisher, 1976; Elwood *et al*., 1981; Meyer & Johnson, 1983; Suberkropp & Chauvet, 1995; Rosemond *et al*., 2002) yet often, neither nutrient was limiting (Triska & Sedell, 1976; Elwood *et al*., 1981; Gessner & Chauvet, 1994; Gessner, Suberkropp & Chauvet, 1997; Royer & Minshall, 2001).

Few studies have looked specifically at nutrient availability and its effect on wood biofilms. Wood biofilms may be even more susceptible than leaf decomposers to nutrient limitation because of the lower nutrient content and higher lignin : nitrogen ratios in the substratum (Melillo *et al*., 1983). In our study, fungi were more frequently nutrient limited than were algae, and six out of 10 streams displayed some form of nutrient limitation (Table 4). Previous research strongly suggests that the rate of wood decomposition (Aumen, Bottomley & Gregory, 1985) and heterotrophic activity are highest (Tank & Winterbourn, 1995) with the addition of N and P. However, wood decomposers can also be limited by the quality of their carbon source. For example, lignin to nitrogen ratios of organic matter influence decomposition rates (Melillo, Aber & Muratore 1982; Melillo *et al*., 1983; Peterson *et al*., 1993).

The pattern of nutrient limitation in heterotrophs may depend on the interaction between microbes colonising different organic substrata. A decrease in both N and P in stream water during autumn has been attributed to uptake by heterotrophic decomposers associated with the autumnal pulse of leaf litter (Newbold *et al*., 1983; Mulholland *et al*., 1985; Mulholland & Rosemond, 1992). But Tank & Webster (1998) found that the presence of decomposing leaf litter in autumn caused a concomitant limitation of wood biofilms, and NDS bioassays showed a colimitation of wood biofilms by both N and P when decomposing leaf litter is present, suggesting that competition for both N and P may regulate heterotrophic microbial processes in forested streams. Similarly, both fungi and algae are part of the biofilm on wood veneers and our data allow us to compare how nutrients affect different functional members of the same biofilm assemblage. Do the heterotrophic and autotrophic components of biofilms respond similarly to nutrient addition when other environmental variables are held constant (e.g. temperature, substratum type)? We found that the potential magnitude of the biomass response was different between autotrophs and heterotrophs; overall the addition of N (N : C or NP : C treatments) gave the greatest increase in biomass, whereas the P response was distinctly different and only fungi respond positively (Fig. 5). Patterns of nutrient limitation usually differed between algae and fungi growing on wood in the same stream. If we distinguish between N limitation and N with secondary P limitation, only three of nine streams were the same, and two of these three showed no limitation. Alternatively, if N limitation and N with secondary P limitation are treated as a similar responses (i.e. an N response of some sort), the number of streams with identical responses increases to five out of nine. Our results show that the heterotrophic and autotrophic components of the biofilm may be limited by different nutrients even though they are experiencing the same stream nutrient conditions.

Can nutrient limitation be predicted from the N/P ratio of stream water?

Rather than an absolute concentration of nutrients, the ratio of N to P has frequently been used as a predictor of nutrient limitation in aquatic systems. A molar ratio of DIN : SRP ≥16–20 may result in conditions where P is limiting (Elwood *et al*., 1981; Peterson *et al*., 1993; Pringle, 1987) while a DIN : SRP ratio <10 has indicated limitation by N (Gregory, 1980; Grimm & Fisher, 1986). For the 10 streams studied here, the biomass response to added N on GF/Fs was negatively correlated with the molar DIN : SRP ratio (Fig. 6), yet the DIN : SRP ratio was only marginally predictive of whether or not wood biofilms would show a statistically significant response to nutrient addition. Other stream studies have had similar difficulty in using the DIN : SRP ratio to predict nutrient limitation (Wold & Hershey, 1999). For example, N limitation could occur at a DIN : SRP ratio ranging from 4 : 1 to 400 : 1 (Francoeur *et al*., 1999), suggesting that the N : P ratio provides information only on whether a nutrient *might* be limiting. We believe that our results may not have followed predicted patterns because most of our streams have a low concentration of both SRP and DIN (Table 2). At such low concentrations, the ratio of DIN : SRP becomes less meaningful. For example, one might not be surprised to find that biofilms are limited by both N and P even when the

N : P ratio is 20 : 1 if DIN is 10 μg N L^{-1} and SRP is 0.5 μg P L^{-1}. Despite a DIN : SRP >16, the absolute quantity of either nutrient that is biologically available is very small.

As an alternative to the hypothesis that nutrient ratios can predict nutrient limitation in streams, perhaps there are threshold values of nutrients below which one might find nutrient limitation. Grimm & Fisher (1986) implied that a NO3-N concentration below 55 μg L^{-1} in Sycamore Creek, AZ would suggest N limitation and our bioassay in that stream did indeed show N limitation at a NO3-N concentration of 9 μg L^{-1}. Yet the threshold hypothesis was not universally supported in our study. A number of streams with a DIN concentration below 55 μg L^{-1} did not show significant N limitation. A higher rate of nutrient recycling within biofilms has been shown to accompany low nutrient concentrations and this may explain why some streams did not show a response to added nutrients (Mulholland *et al*., 1991). In lotic systems, continuous unidirectional flow might cause deviation from expected nutrient limitation patterns related to concentration. For example, if there is a continuous flux, and therefore delivery, of nutrients across any biofilm surface, biofilm nutrient requirements can be met despite low nutrient concentrations in stream water. We believe this issue of nutrient flux and delivery in streams and rivers merits further study (Borchardt, 1996).

Table 5 Literature review of nutrient diffusing substrata experiments. N or P mean apparent N or P limitation, respectively, NXP means a colimitation by both N and P or primary limitation by one with secondary limitation by the other. Statistics supplied in the paper were used when available, otherwise differences exceeding the error bars provided were assumed significant

	Chl response (apparent limitation)				
Site	None	N	P	NXP	Citation
French R., Minnesota, two seasons		1	1	2	Allen & Hershey (1996)
Kiakuni R., New Zealand, four seasons	7			5	Biggs, Kilroy & Lowe (1998)
Two rivers, Michigan			1	1	Burton *et al*. (1991)
Seven Australian streams, summer	1	2	1	3	Chessman, Hutton & Burch (1992)
Seven Australian streams, autumn	1	2	3	1	Chessman *et al*. (1992)
Six streams, Ontario			6		Corkum (1996)
10 New Zealand streams, summer	1	1	1	7	Francoeur *et al*. (1999)
11 New Zealand streams, autumn	5			6	Francoeur *et al*. (1999)
11 New Zealand streams, winter	7	1		3	Francoeur *et al*. (1999)
11 New Zealand streams, spring	3	6		2	Francoeur *et al*. (1999)
Wah Umkhen R., India			4		Ghosh & Gaur (1994)
Sycamore Creek, Arizona		1			Grimm & Fisher (1986)
Bradley Brook, New York, autumn			2		Hepinstall & Fuller (1994)
Two streams, California		1		1	Hill & Knight (1988)
Three rivers, Minnesota	2		1		Kutka & Richards (1997)
Saline Creek, Missouri		1			Lohman *et al*. (1991)
Mary R. catchment, Australia		1			Mosisch *et al*. (1999)
Nechako R., British Columbia		1			Perrin & Richardson (1997)
Carnation Creek, British Columbia			1		Pringle (1987)
Carp Creek, Michigan			1		Pringle & Bowers (1984)
Athabasca R., Ontario, CA, autumn			3		Scrimgeour & Chambers (1997)
Guadeloupe R., TX, three seasons			2	1	Stanley *et al*. (1990)
Kings Creek, Kansas				1	Tate (1990)
Middle Bush, New Zealand, winter				2	Winterbourn (1990)
East Split Rock R., Minnesota, 12 mo	2	1	1	7	Wold & Hershey (1999)
Encampment R., Minnesota, 12 mo	4	1	2	5	Wold & Hershey (1999)
Knife R., Minnesota, 12 mo	2		2	8	Wold & Hershey (1999)
Silver Creek, Minnesota, 12 mo	3	1		8	Wold & Hershey (1999)
Stewart R., Minnesota, 12 mo	2		4	6	Wold & Hershey (1999)
West Split Rock R., Minnesota, 12 mo	5		2	5	Wold & Hershey (1999)
Total (%)	45 (25)	21 (12)	38 (22)	72 (41)	

How common is colimitation in streams?

Considering broad patterns of N and P availability, algal growth in lakes is usually assumed to be limited by P supply (Hecky & Kilham, 1988) or colimited by P and N (Elser, Marzolf & Goldman, 1990; Dodds, Johnson & Priscu, 1989). Is this also true for streams? In a literature review of previously published NDS studies (all for epilithon in lotic systems), we found that limitation of algal biomass by both N and P together was reported most frequently (41% of occasions, Table 5). The next most common response was no response to N or P addition (25%), followed by 22% occurrence of solely P limitation, and N limitation alone reported for 12% of the cases (Table 5). Using meta-analysis of lotic NDS studies that resulted in increased statistical power, Francoeur (2001) found that simultaneous stimulation of algae by >1 nutrient (e.g. both N and P) was the most frequent response and concluded that multispecies biofilm communities are unlikely to be limited by just one nutrient. The results from our study are consistent with these general trends from previous studies and indicate that sole P limitation, despite very low apparent SRP concentrations, seems less frequent in streams than in lakes, even for the heterotrophic biofilms not previously studied in this context. In fact, we never saw algal limitation by P alone and it occurred only twice for the fungi on wood (Table 4). Why is colimitation by N and P so common? Previous research has shown that algal species differ in the nutrient requirements (e.g. Borchardt, 1996) and it follows that species-specific nutrient requirements could exist for heterotrophic microbes as well. In a multispecies biofilm, be it heterotrophic, autotrophic or a mix of the two, different species can be limited by different nutrients. Additionally, when N and P concentrations are very low, there may be an additive effect of N + P amendment. Adding N or P alone would not affect biofilm biomass, but the addition of the primary limiting nutrient depletes the secondary limiting nutrient sufficiently quickly for both nutrients to limit growth (Tate, 1990; Francoeur, 2001).

Our results suggest that different nutrients or combinations of nutrients may limit heterotrophic and autotrophic components of stream biofilms and these differences may vary spatially across habitats and substrata. When designing nutrient enrichment bioassays, substrata should be chosen that are as similar as possible to the dominant substratum type in the stream. Two lines of future research could improve our understanding of nutrient-biofilm relationships in streams: (1) further research into the geographic distribution of N and P limitation and (2) taxonomic composition of biofilms (including bacteria) and how it might change with nutrient addition.

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