

# Seasonal variation in nutrient limitation of microbial biofilms colonizing organic and inorganic substrata in streams

Timothy J. Hoellein · Jennifer L. Tank ·  
John J. Kelly · Emma J. Rosi-Marshall

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**Abstract** Humans have increased the availability of nutrients including nitrogen and phosphorus worldwide; therefore, understanding how microbes process nutrients is critical for environmental conservation. We examined nutrient limitation of biofilms colonizing inorganic (fritted glass) and organic (cellulose sponge) substrata in spring, summer, and autumn in three streams in Michigan, USA. Biofilms were enriched with nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ),  $\text{NO}_3^- + \text{PO}_4^{3-}$ ,  $\text{NH}_4^+ + \text{PO}_4^{3-}$ , or none (control). We quantified biofilm structure and function

as chlorophyll *a* (i.e., primary producer biomass) and community respiration on all substrata. In one stream, we characterized bacterial and fungal communities on cellulose in autumn using clone library sequencing and denaturing gradient gel electrophoresis to determine if community structure was linked to nutrient limitation status. Despite oligotrophic conditions, primary producer biomass was infrequently nutrient limited. In contrast, respiration on organic substrata was frequently limited by N + P combinations. We found no difference between biofilm response to  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  enrichment, although the response to both N-species was positively related to water column  $\text{PO}_4^{3-}$  concentrations and temperature. Molecular analysis for fungal community composition suggested no relationship to

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T. J. Hoellein · J. L. Tank  
Department of Biological Sciences, University of Notre Dame, 188 Galvin Hall, Notre Dame, IN 46556, USA  
e-mail: jtank@nd.edu

T. J. Hoellein (✉)  
Department of Natural Sciences, Baruch College, City University of New York, Box A-506, 17 Lexington Ave, New York, NY 10010, USA  
e-mail: timothy.hoellein@baruch.cuny.edu

J. J. Kelly · E. J. Rosi-Marshall  
Department of Biology, Loyola University of Chicago, 1032 W. Sheridan Rd., Chicago, IL 60626, USA

J. J. Kelly  
e-mail: jkelly@luc.edu

J. J. Kelly · E. J. Rosi-Marshall  
Center for Urban Environmental Research and Policy, Loyola University Chicago, 1032 W. Sheridan Rd., Chicago, IL 60626, USA

*Present Address:*  
E. J. Rosi-Marshall  
Cary Institute of Ecosystem Studies, 2801 Sharon Turnpike Box AB, Millbrook, NY 12545, USA

E. J. Rosi-Marshall  
e-mail: rosimarshalle@caryinstitute.org

nutrient limitation, but the dominant members of the bacterial community on cellulose were different on  $\text{NO}_3^-$ ,  $\text{PO}_4^3-$ , and  $\text{NO}_3^- + \text{PO}_4^3-$  treatments relative to control,  $\text{NH}_4^+$ , and  $\text{NH}_4^+ + \text{PO}_4^3-$  treatments, which matched patterns for biofilm respiration rates from each treatment. Our results show discrete patterns of nutrient limitation dependent upon substratum type and season, and imply changes in bacterial community structure and function may be linked following nutrient enrichment in streams.

**Keywords** Stream biofilms · Nitrogen · Phosphorus · Ecosystem metabolism · Bacteria · Fungi · DGGE · DNA

## Introduction

The availability of nutrients is increasing globally (Vitousek et al., 1997), resulting in eutrophication of aquatic ecosystems including rivers, lakes, and estuaries (Paul & Meyer, 2001; Rabalais et al., 2002). For example, in the agricultural Midwestern United States, high inorganic nitrogen (N) and phosphorus (P) levels are of concern in most flowing waters (Alexander et al., 2000). Because N and/or P commonly limit growth of stream biota at the base of the food web (e.g., algae, bacteria, and fungi), nutrient enrichment can have significant effects on community structure and function (Tank & Dodds, 2003; Cross et al., 2006). The ability of stream microorganisms to process nutrients is of foremost interest for maintaining high water quality and mitigating negative effects of eutrophication on ecological health.

Biofilms are the major mechanism for assimilation of inorganic nutrients into stream food webs. Aquatic biofilms typically have mixtures of autotrophic (i.e., algae and bacteria) and heterotrophic (i.e., bacteria and fungi) constituents that are able to take up inorganic nutrients from the surrounding environment. In stream ecosystems, both autotrophic and heterotrophic assimilation have bottom-up effects on food webs (Rosemond et al., 1993; Hall & Meyer, 1998). The relative importance of autotrophic and heterotrophic biofilm components to higher trophic levels is geographically and temporally variable (Webster et al., 2003). However, nutrient limitation of heterotrophic biofilms is rarely assessed (Tank & Dodds, 2003), because most studies use inorganic substrata in nutrient limitation

assays, which selects for a largely autotrophic community (Johnson et al., 2009). As a result, there is a lack of empirical data comparing nutrient limitation of biofilms colonizing inorganic versus organic substrata, which is critical for our general understanding of stream ecosystem function, and for generating predictions regarding the effects of eutrophication in different streams, biomes, and seasons.

In temperate biomes, seasonality may affect the response of biofilms to changes in nutrient availability (Roberts et al., 2007). For example, seasonal changes in light availability have been shown to more strongly limit the growth of autotrophic biofilms than nutrient availability (Mosisch et al., 2001). In forested headwater streams, light availability to the streambed varies by season, potentially altering the relative importance of nutrient availability to biofilm growth and metabolism (Roberts et al., 2007). Similarly, carbon (C) available for heterotrophic microbial colonization (e.g., leaves) peaks in autumn, which can stimulate demand for inorganic nutrients as heterotrophs decompose the leaf litter (Hoellein et al., 2009). Seasonal variation in nutrient limitation status on biofilms colonizing different substrata in forested headwater streams has not been previously measured, and represents a gap in our understanding of the interaction between seasonality and nutrient processing by autotrophic and heterotrophic constituents of stream biofilms.

In addition to functional metrics such as rates of nutrient cycling and metabolism, nutrient enrichment can affect biofilm community composition (Pringle, 1990; Cross et al., 2006). Understanding how microbial diversity relates to ecosystem function in the context of shifting resources (i.e., changes in nutrient or C availability) is a major goal of current microbial ecology research. A majority of the research using molecular analyses of lotic microbial communities has focused on biogeography (Glockner et al., 2000; Brummer et al., 2003; de Figueiredo et al., 2007; Rubin & Leff, 2007), and has less often addressed changes in community composition following experimental manipulations of biofilm resources (but see Olapade & Leff, 2005; Das et al., 2007). In streams, understanding the relationship between diversity and function is crucial because microbial communities compose the base of the food web and are critical for the biogeochemical cycling of C and nutrients (Hall & Meyer, 1998). A goal of this research was to

couple molecular analysis of microbial communities [clone library sequencing paired with denaturing gradient gel electrophoresis (DGGE)] with a functional metric (microbial community respiration) following nutrient enrichment of an organic substratum. This approach is unique because molecular tools have rarely been applied to elucidate response of heterotrophic biofilms to experimental eutrophication in stream ecosystems.

We used nutrient diffusing substrata (NDS) to address three objectives in this study: (1) to compare nutrient limitation patterns of stream biofilms in three forested streams in northern Michigan in spring, summer, and autumn, on two substrata, organic (cellulose sponge) and inorganic (fritted glass disks), using structural (i.e., chlorophyll *a* to indicate primary producer biomass) and functional [i.e., community respiration (CR)] metrics, (2) to quantify the effect of external drivers such as temperature, light, discharge, and water column nutrients on seasonal variability in nutrient limitation status, and (3) to document whether changes in functional response (i.e., CR) correspond to changes in the microbial community composition of cellulose biofilms after enrichment.

## Methods

### Study sites

State, Shane, and Walton Creeks are forested, first-order streams in the Ontonagon River basin of Lake Superior, in the Upper Peninsula of Michigan, USA. All three streams are located in the Ottawa National Forest, and land-use within the stream watersheds is primarily forested (83–95%), with low human and wetland influence (Entrekin et al., 2007). The climate is characterized by mild summers, mean July air temperature is 18°C, and cold winters, and mean January air temperature is –10°C (Hoellein et al., 2007). We chose these streams to represent “reference” conditions for impacted agricultural streams in the upper Midwestern US that have undergone eutrophication due to agricultural influences because these streams are oligotrophic with regard to inorganic nutrient concentrations (Table 1). Riparian vegetation consists of second-growth mixed hardwood forest with the dominant species including white pine (*Pinus*

*strobes* L.), eastern hemlock (*Tsuga canadensis* L.), sugar maple (*Acer saccharum* Marsh.), red maple (*Acer rubrum* L.), alder (*Alnus* spp.), and paper birch (*Betula papyrifera* Marsh.), with an understory consisting of mixed grasses and ferns.

### Experiment 1: seasonal variation of nutrient limitation

We constructed NDS following the method described by Johnson et al. (2009). NDS were made from 30 ml plastic cups filled with 2% agarose amended with five treatments: nitrate (0.5 M  $\text{NaNO}_3^-$ ), phosphate (0.5 M  $\text{KH}_2\text{PO}_4^-$ ), ammonium (0.5 M  $\text{NH}_4^+\text{Cl}$ ),  $\text{NO}_3^- + \text{PO}_4^{3-}$ , and  $\text{NH}_4^+ + \text{PO}_4^-$ . Combined treatments contained 0.5 M of both N and P. Cups were capped with either fritted glass disks or cellulose sponge. We deployed five replicates of each treatment type plus a control group (e.g., no nutrients added to agar) in each of the three study streams ( $N = 30$  NDS per stream per substratum) during three seasons: summer (Jul. 24–Aug. 15, 2003), autumn after leaf-fall (Nov. 22–Dec. 13, 2003), and spring prior to leaf-out (Apr. 18–May 4, 2004). We attached the NDS to a plastic L-bar in random order, and then nailed the L-bars to the stream benthos in a riffle area. After ~23 days incubation, we placed each substratum in a 50 ml centrifuge tube with stream water, which were placed on ice for transport back to the laboratory. NDS incubation times are typically slightly shorter (Johnson et al., 2009), however, NDS constructed of these materials continue to diffuse solutes for >28 days (Tank et al., 2006).

Chlorophyll *a* (chl *a*) and CR were measured on all substrata to indicate biofilm structure (i.e., primary producer biomass) and function, respectively. Within 24 h of retrieval from the study sites, we filled each centrifuge tube containing one substratum with unfiltered stream water of known dissolved oxygen (DO), using care to eliminate any air bubbles. For each stream we included three “blank” tubes, which contained only water to correct for any background changes in DO. We incubated tubes on a shaker table in the dark for 2 h and recorded the final DO. We then extracted the substrata for chl *a* using the non-acidification, hot ethanol method (Sartory & Grobbelaar, 1984) and a Turner Designs Model

**Table 1** Mean ( $\pm$ SE) temperature, discharge, depth, canopy cover, water column concentrations of ammonium ( $\text{NH}_4^+$ ), soluble reactive phosphorus (SRP), and nitrate ( $\text{NO}_3^-$ )

Season	Stream	Temperature ( $^{\circ}\text{C}$ )	Discharge ( $\text{l s}^{-1}$ )	Depth (cm)	Canopy cover (%)	$\text{NH}_4^+$ ( $\mu\text{gN l}^{-1}$ )	SRP ( $\mu\text{gP l}^{-1}$ )	$\text{NO}_3^-$ ( $\mu\text{gN l}^{-1}$ )
Spring	State	6.5 (0.2)	83 (4)	15.5 (1.0)	42.0 (2.6)	3.8 (0.2)	4.8 (0.1)	164.9 (1.1)
	Shane	6.9 (0.2)	98 (12)	5.8 (1.0)	46.6 (2.5)	6.2 (0.6)	6.4 (0.1)	79.2 (2.6)
	Walton	9.6 (0.4)	56 (3)	12.3 (0.1)	33.1 (1.9)	3.6 (0.2)	5.6 (0.7)	263.3 (34.3)
Summer	State	11.7 (0.1)	47 (1)	5.8 (0.6)	87.0 (2.5)	5.1 (6.7)	6.7 (0.4)	131.4 (0.6)
	Shane	15.0 (<0.1)	27 (1)	8.4 (1.2)	86.3 (2.3)	6.3 (1.0)	9.8 (1.2)	186.4 (1.3)
	Walton	11.7 (0.1)	27 (0.4)	5.7 (1.0)	85.3 (0.7)	5.8 (0.3)	6.6 (0.2)	627.9 (13.4)
Autumn	State	2.5 (0.1)	52 (1)	6.4 (0.3)	54.5 (3.9)	8.1 (1.7)	2.2 (0.1)	170.6 (0.2)
	Shane	1.0 (<0.1)	26 (2)	22.7 (0.5)	60.3 (3.7)	14.6 (0.4)	6.2 (0.6)	239.4 (7.1)
	Walton	3.1 (0.1)	30 (1)	14.8 (0.5)	36.8 (2.7)	4.3 (0.1)	3.5 (0.1)	308.2 (33.8)

Temperature and discharge are the mean values for the incubation period, while the other parameters were measured on the collection date. Water chemistry in autumn in Walton Creek was collected in October 2003 (1.5 months prior to the data from State and Shane Creeks)

TD-700 Fluorometer. Finally, we measured ash-free dry mass (AFDM) of biofilms on organic and inorganic biofilms. We calculated CR as the change in  $\text{O}_2$  per substratum area per time or as change in  $\text{O}_2$  per gAFDM per time (Hill et al., 2002).

We quantified several stream physiochemical and biological variables as potential predictors of NDS response. We measured depth of the NDS as the mean distance from the water surface to the substratum at four corners and the center of the L-bars. Forest canopy cover was estimated using a spherical densitometer. We calculated mean discharge during the incubation period from a regression between the discharge of each stream and a downstream USGS gauging station on the Ontonagon River. Finally, data-loggers recorded stream temperature every hour during NDS incubation periods.

We analyzed solute concentrations from water samples collected in 60 ml acid-washed nalgene bottles from 10 stations distributed  $\sim 100$  m upstream and  $\sim 100$  m downstream of NDS on the incubation period's final day. Intraseasonal variation of N and P concentrations is low in these sites (Hoelein et al., 2007); however, we did not measure organic N or P. We filtered samples in the field using glass fiber filters (0.7  $\mu\text{m}$  nominal pore size), and froze them until solute analysis. We used ion chromatography with AS14A analytical and guard columns and a 500  $\mu\text{l}$  injection loop to measure  $\text{NO}_3^-$  concentrations (USEPA 1993). Ammonium ( $\text{NH}_4^+$ ) was measured using the phenyl-hypochlorite

technique (Solorzano, 1969), and  $\text{PO}_4^{3-}$  was measured as soluble reactive phosphorus (SRP) with the molybdate–antimony method (Murphy & Riley, 1962).

#### Experiment 2: molecular analysis of enriched cellulose biofilms

We incubated NDS with cellulose after leaf-fall (autumn) for 28 days in State Creek, from Nov. 7 to Dec. 5, 2006 (3 years after Experiment 1). We selected State Creek, autumn, and cellulose because that is when nutrient limitation was most strongly recorded in previous experiments. We used the same nutrient treatments and measured CR as described above. Immediately after quantifying respiration, one cellulose sponge from each treatment ( $N = 6$ ) was cut into small pieces using sterile forceps and surgical scissors, and pieces were placed in a centrifuge tube and frozen at  $-80^{\circ}\text{C}$  until DNA extraction. Thirty-six hours later, DNA was extracted from the cellulose using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). Our approach to DGGE, cloning, and sequencing was somewhat modified (but see Green et al., 2007). First, we created a clone library of the DNA amplified from the cellulose biofilm for each nutrient treatment. Next, we used DGGE to identify the clones representing the major taxa in each of the environmental communities (Muyzer & Smalla, 1998). Finally, the clones representing the major taxa were sequenced.

## PCR and cloning

Bacterial 16S rRNA genes were amplified from extracted DNA using universal primers EUB341F-GC which has a GC-rich clamp and is specific to most bacteria, and EUB534R (Muyzer et al., 1993). To amplify fungal ribosomal intergenic transcribed spacer region 2 (ITS2), we used primers ITS3-GC, which has a GC-rich clamp (Nicolcheva et al., 2005), and ITS4 (White et al., 1990). Each 50  $\mu$ l PCR reaction contained 0.4  $\mu$ M forward and reverse primers, 2.5 mM MgCl<sub>2</sub>, 160  $\mu$ M dNTPs, 1 $\times$  GoTaq buffer, 3 units GoTaq DNA polymerase, and 3  $\mu$ l environmental DNA, diluted 1:10 from the extraction. All PCR reactions were performed using a thermocycler and initial denaturing temperature of 94°C for 2 min, followed by 40 cycles of denaturing at 94°C for 1 min, annealing at 56.4°C for 1 min, and extension at 72°C for 1 min. The final extension period was 5 min at 72°C. Bacterial and fungal PCR amplicons were cloned with the TOPO-TA cloning kit using PCR-2.1 vector and were transformed into chemically competent *Escherichia coli*. Transformed *E. coli* were grown overnight on LB agar plates with 50  $\mu$ g ml<sup>-1</sup> kanamycin. Randomly selected colonies were transferred to LB broth containing 50  $\mu$ g ml<sup>-1</sup> kanamycin, grown overnight at 37°C, and PCR-screened for the presence of inserts using M13F and M13R primers. Plasmids containing the insert were isolated using the Mini Plasmid Prep Kit (MoBio Laboratories, Inc.).

## DGGE and sequencing

We next used DGGE to identify the clones that represented the major taxa in the environmental community. First, we amplified each cloned plasmid using PCR with DGGE primers. We ran the PCR product from each clone on a DGGE gel where the middle lane contained the PCR product from the original environmental sample (also amplified with DGGE primers). If a cloned band lined up with a band in the environmental sample, we counted it as a major part of the community, and we sequenced it (for example, see Appendix Fig. 1—Supplementary material). If a cloned band migrated to a position on the DGGE gel that was not in line with a band in the environmental sample, we considered this a *minor* member of the community, and did not sequence it

(Green et al., 2007). We repeated this approach for each nutrient amendment. Selected clones were sequenced bidirectionally using the M13F and M13R primers at the University of Chicago Cancer Research Center's DNA Sequencing Facility.

DGGE was conducted using the Biorad D-Code Universal Mutation Detection System. Gels contained 6% (for fungal amplicons) or 8% (for bacterial amplicons) polyacrylamide (weight/volume). The urea–formamide gradient was 30–60% (bacterial amplicons) or 20–60% (fungal amplicons). We cast the DGGE gel with a gradient maker and pump and electrophoresis was conducted at 60 V for 14–16 h, or at 45 V for 16–18 h at 60°C (Kominoski et al., 2009). We stained gels with GelStar nucleic acid stain and imaged them with a digital gel imaging system. All PCR and DGGE were conducted using negative (no DNA) and positive controls for bacteria or fungi, *Psudeomonas aeruginosa* and *Cotinarius multiformas*, respectively.

## Data analysis

We used a two-way repeated measures analysis of variation (RM-ANOVA) to compare differences in physiochemical factors among dates and streams. For control substrata (no nutrients added), we used a three-way RM-ANOVA to compare chl *a* (i.e., primary producer biomass) and CR on fritted glass by substratum type, date, and stream. Following significant interactions, we tested for the influence of each factor separately using a Bonferonni-corrected *P* value for substratum type (*t* test,  $P = 0.05/9 = 0.006$ ), date (RM-ANOVA,  $P = 0.05/3 = 0.017$ ), and stream (ANOVA,  $P = 0.05/3 = 0.017$ ; Zar, 1999).

To test for nutrient limitation, we used an ANOVA with two factors, the presence of N or P (Tank & Dodds, 2003). We performed first two-way ANOVA for comparison among control, NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, and NO<sub>3</sub><sup>-</sup> + PO<sub>4</sub><sup>3-</sup>, and a second two-way ANOVA to compare among control, NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>3-</sup>, and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3-</sup>. We log-transformed the data prior to analysis if it did not meet assumptions of ANOVA. Using Tank & Dodds (2003) protocol for NDS analyses, limitation was indicated when NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, or NH<sub>4</sub><sup>+</sup> alone resulted in a positive response without a significant interaction. Co-limitation was indicated when two treatments independently affected the response, or when a combined treatment

( $\text{NO}_3^- + \text{PO}_4^{3-}$  or  $\text{NH}_4^+ + \text{PO}_4^{3-}$ ) significantly increased the response. We used linear regression of physiochemical parameters with the nutrient response ratio (i.e.,  $\log[\text{treatment/control}]$ ; NRR) for chl *a* and respiration on each substratum type across all streams and dates ( $N = 9$ ; Tank & Dodds, 2003). Because nutrient limitation patterns of respiration rates expressed as AFDM were largely identical to those expressed in terms of surface area, we present areal rates only here. We completed all statistics with Systat 11.0.

The SeqMan component of Lasergene software was used to assemble a consensus sequence for each clone. Bacterial clone sequences were deposited to Genbank under accession numbers EU709496–EY709512 and fungal clone sequences were deposited under accession numbers EU709513–EU709516. We compared our sequences to those in the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul et al., 1990). Closely related sequences were downloaded into Megalign, sequences were aligned using ClustalW, and phylogenetic trees were created with Jukes-Cantor neighbor joining and bootstrapping.

## Results

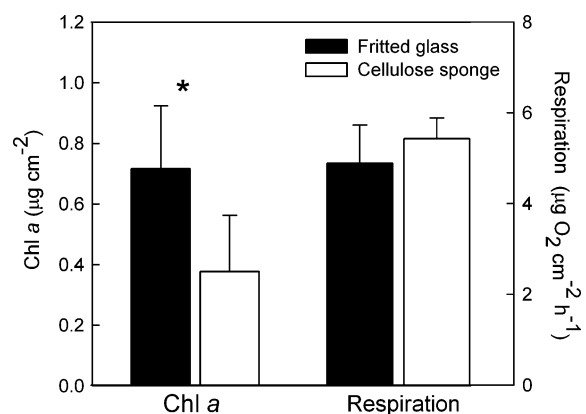
### Experiment 1: seasonal variation in physiochemical parameters

We found strong seasonal differences in the physical, chemical, and biological characteristics of the stream environments during the NDS incubation periods, which were consistent with our expectations for the temperate climate of the region. In all streams, we found the highest values in the summer for canopy cover (RM-ANOVA,  $P < 0.01$ ), temperature (RM-ANOVA,  $P < 0.01$ ), and SRP concentrations (RM-ANOVA,  $P < 0.01$ ), while discharge was highest in the spring (RM-ANOVA,  $P < 0.01$ ).

On control substrata, we used a three-way RM-ANOVA with chl *a* or respiration as dependent variables, and substratum type, date, and stream as factors. We found significant interactions among the three factors, so we analyzed each factor separately using Bonferonni-corrected  $P$  values. We found higher chl *a* density on fritted glass relative to cellulose sponge ( $t$  test,  $P = 0.005$ ), indicating

greater density of autotrophic biomass on fritted glass, but no difference in respiration rates between substrata ( $P = 0.35$ ; Fig. 1). For each date, primary producer biomass was always higher on fritted glass relative to cellulose sponge in summer (ANOVA,  $P < 0.001$ ), while CR was always higher on cellulose sponge relative to fritted glass in the spring (ANOVA,  $P < 0.001$ ). By stream, we found Shane Creek had the lowest primary producer biomass in autumn (RM-ANOVA,  $P < 0.001$ ), and Walton Creek had the highest primary producer biomass in spring (RM-ANOVA,  $P < 0.001$ ). For CR, Walton Creek had the highest rates in autumn (RM-ANOVA,  $P < 0.001$ ), and Shane Creek was highest in summer (RM-ANOVA,  $P < 0.001$ ).

Overall, we found few instances of nutrient limitation on fritted glass, and where nutrient limitation was found, the patterns were different among streams and seasons (Table 2, Fig. 2). There were no cases where the same nutrient was limiting primary producer biomass in all three replicate streams, and there was no season where one of the streams was not nutrient limited. For the nine potential occurrences of nutrient limitation of primary producer biomass on glass (i.e., three streams and three dates), significant nutrient limitation occurred only three times. All three cases were distinct, including limitation by P alone (Walton, spring), N-limitation (as  $\text{NH}_4^+$ ; State Creek, summer), or co-limitation of N and P (as  $\text{NH}_4^+$ ; State Creek, autumn). Significant limitation of

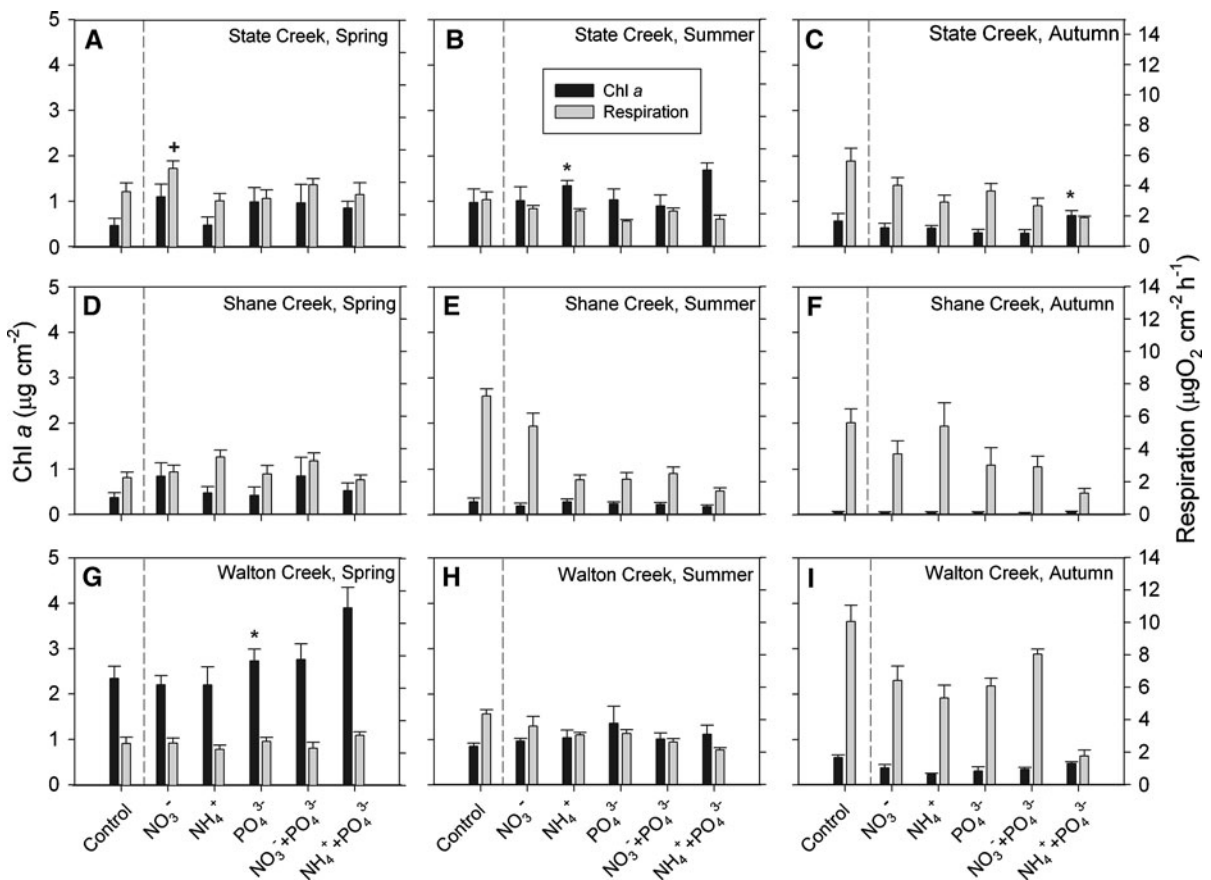


**Fig. 1** Chlorophyll *a* (chl *a*) and microbial respiration on fritted glass and cellulose sponge for control substrata (no nutrients added). Chl *a* was significantly different between substrata ( $t$  test,  $P = 0.005$ ), but respiration was not ( $t$  test,  $P = 0.351$ )

**Table 2** Nutrient limitation indicated by nutrient diffusing substrata for chlorophyll *a* (Chl *a*) and respiration on fritted glass and cellulose sponge substrata in State, Shane, and Walton Creeks in spring, summer, and autumn

Season	Stream	Fritted glass		Cellulose sponge	
		Chl <i>a</i>	Respiration	Chl <i>a</i>	Respiration
Spring	State	–	NO <sub>3</sub> <sup>–</sup>	–	PO <sub>4</sub> <sup>3–</sup>
	Shane	–	–	–	NO <sub>3</sub> <sup>–</sup> + PO <sub>4</sub> <sup>3–</sup>
	Walton	PO <sub>4</sub> <sup>3–</sup>	–	–	NH <sub>4</sub> <sup>+</sup> + PO <sub>4</sub> <sup>3–</sup> , NO <sub>3</sub> <sup>–</sup> + PO <sub>4</sub> <sup>3–</sup>
Summer	State	NH <sub>4</sub> <sup>+</sup>	–	–	NO <sub>3</sub> <sup>–</sup> + PO <sub>4</sub> <sup>3–</sup>
	Shane	–	–	NH <sub>4</sub> <sup>+</sup>	–
	Walton	–	–	PO <sub>4</sub> <sup>3–</sup>	–
Autumn	State	NH <sub>4</sub> <sup>+</sup> + PO <sub>4</sub> <sup>3–</sup>	–	–	NH <sub>4</sub> <sup>+</sup> + PO <sub>4</sub> <sup>3–</sup>
	Shane	–	–	NH <sub>4</sub> <sup>+</sup> + PO <sub>4</sub> <sup>3–</sup>	–
	Walton	–	–	–	–

NO<sub>3</sub><sup>–</sup>, nitrate alone; PO<sub>4</sub><sup>3–</sup>, phosphate alone; NH<sub>4</sub><sup>+</sup>, ammonium alone; NO<sub>3</sub><sup>–</sup> + PO<sub>4</sub><sup>3–</sup>, nitrate and phosphate together; NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3–</sup>, ammonium and phosphate together, and – indicates no limitation



**Fig. 2** Chlorophyll *a* (chl *a*) and respiration on fritted glass amended with no nutrients (control), nitrate (NO<sub>3</sub><sup>–</sup>), phosphate (PO<sub>4</sub><sup>3–</sup>), ammonium (NH<sub>4</sub><sup>+</sup>), NO<sub>3</sub><sup>–</sup> + PO<sub>4</sub><sup>3–</sup>, and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3–</sup> in State, Shane, and Walton Creeks in spring (18 Apr.–14 May 2004), summer (21 Jul.–15 Aug. 2003) and autumn (22

Nov.–13 Dec. 2003). \* indicates significant nutrient limitation for chl *a*, and + indicates significant nutrient limitation among treatments for respiration. Significant limitation calculated after Tank & Dodds (2003)

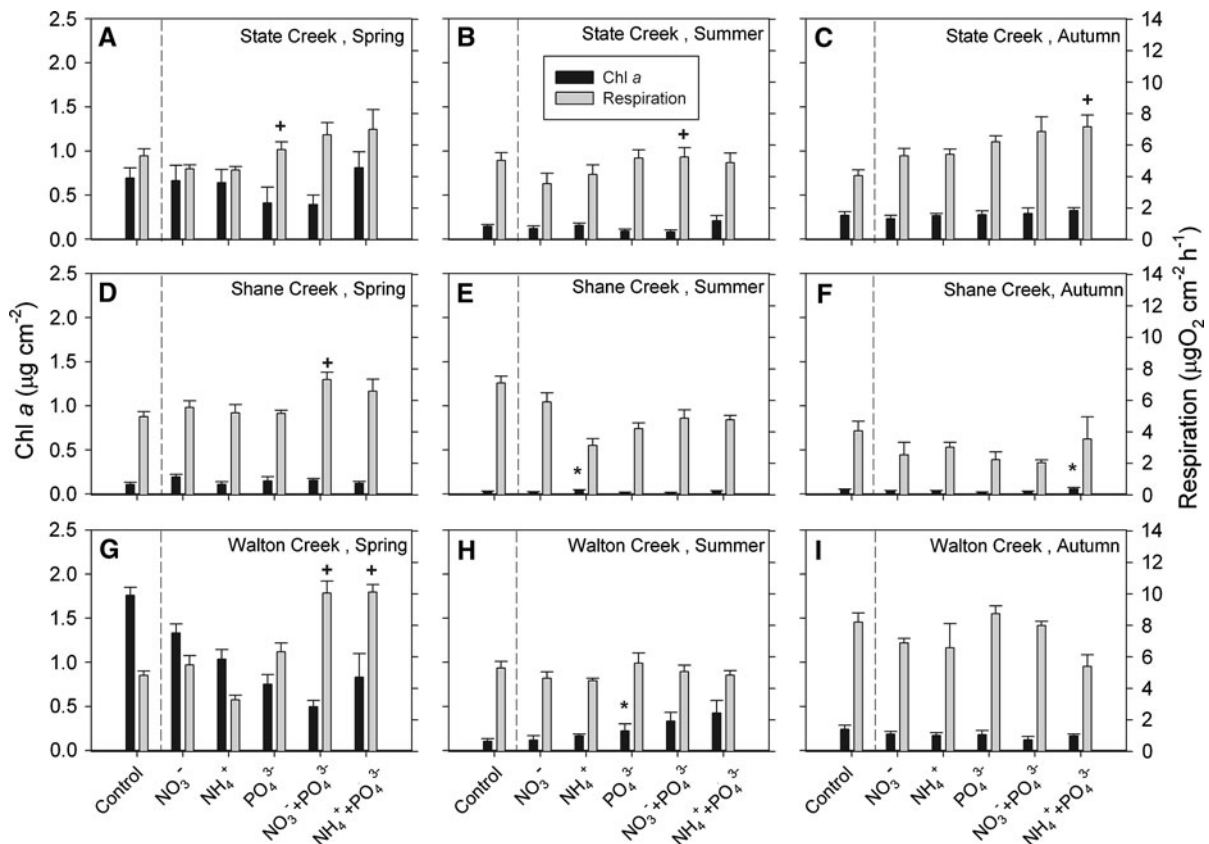
CR on fritted glass occurred in only one out of nine cases: State Creek in spring.

Similar to the results for fritted glass, there were no cases where the same nutrient was limiting primary producer biomass on cellulose (Table 2, Fig. 3). For the nine potential occurrences, significant limitation occurred three times. Each of the results were distinct, including P limitation (Walton Creek, summer), N-limitation (as  $\text{NH}_4^+$ ; Shane Creek, summer), or N and P co-limitation (as  $\text{NH}_4^+$ ; Shane Creek, autumn). For the nine potential occurrences of nutrient limitation of CR on cellulose sponge, significant limitation occurred six times, and in several cases, the same treatment was limiting across streams (Table 2, Fig. 3). P, either alone or in combination with N, was limiting to CR on cellulose in all three streams in the spring. We found N and P co-limitation twice in spring (as  $\text{NO}_3^-$ ; Shane and

Walton Creeks), and once in summer (Walton Creek). In addition, N and P co-limitation (as  $\text{NH}_4^+$ ) was found for CR on cellulose in two cases, once in spring (Walton Creek) and once in autumn (State Creek).

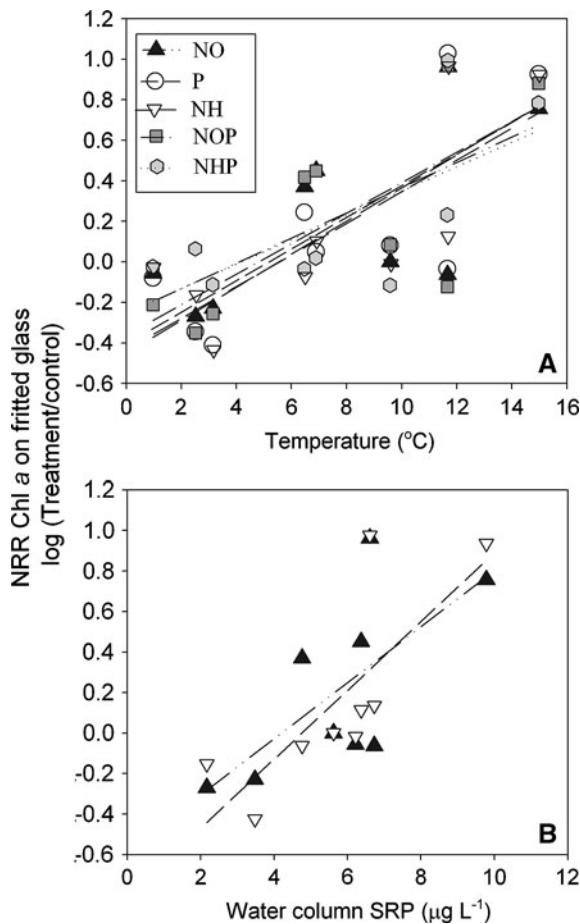
We found inhibition of respiration of inorganic biofilms by N and P in autumn (State and Walton Creeks), summer (Shane and Walton Creeks), and by P in autumn (Shane Creek; Fig. 2C, E, F, H, I). Respiration of cellulose biofilms was inhibited by N and P in autumn (Shane and Walton Creeks) and summer (Shane Creek) Fig. 2E, F, I). Primary producer biomass was N + P co-inhibited in Walton Creek in spring (Fig. 3G).

The NRR was related to several environmental factors for biofilms on the fritted glass, but not for cellulose sponge (Fig. 4). NRR of primary producer biomass on fritted glass was positively related to temperature for  $\text{NO}_3^-$  ( $r^2 = 0.46$ ,  $P = 0.05$ ),  $\text{PO}_4^{3-}$



**Fig. 3** Chlorophyll *a* (chl *a*) and respiration on cellulose sponge amended with no nutrients (control), nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ),  $\text{NO}_3^- + \text{PO}_4^{3-}$ , and  $\text{NH}_4^+ + \text{PO}_4^{3-}$  in State, Shane, and Walton Creeks in spring (18 Apr.–14 May 2004), summer (21 Jul.–15 Aug. 2003) and

autumn (22 Nov.–13 Dec. 2003). \* indicates significant nutrient limitation for chl *a*, and + indicates significant nutrient limitation among treatments for respiration. Significant limitation calculated after Tank & Dodds (2003)



**Fig. 4** Linear regressions between **A** temperature and chlorophyll a (chl *a*) NRR (treatment/control) for NO<sub>3</sub><sup>-</sup> ( $r^2 = 0.46$ ,  $P = 0.05$ ), PO<sub>4</sub><sup>3-</sup> ( $r^2 = 0.60$ ,  $P = 0.002$ ), NH<sub>4</sub><sup>+</sup> ( $r^2 = 0.62$ ,  $P = 0.03$ ), NO<sub>3</sub><sup>-</sup> + PO<sub>4</sub><sup>3-</sup> ( $r^2 = 0.53$ ,  $P = 0.02$ ), and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3-</sup> ( $r^2 = 0.50$ ,  $P = 0.02$ ) and **B** water column soluble reactive phosphorus (SRP) and chl *a* NRR for NO<sub>3</sub><sup>-</sup> ( $r^2 = 0.46$ ,  $P = 0.05$ ) and NH<sub>4</sub><sup>+</sup> ( $r^2 = 0.60$ ,  $P = 0.02$ )

( $r^2 = 0.60$ ,  $P = 0.002$ ), NH<sub>4</sub><sup>+</sup> ( $r^2 = 0.62$ ,  $P = 0.03$ ), NO<sub>3</sub><sup>-</sup> + PO<sub>4</sub><sup>3-</sup> ( $r^2 = 0.53$ ,  $P = 0.02$ ), and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3-</sup> ( $r^2 = 0.50$ ,  $P = 0.02$ ). Primary producer biomass on fritted glass was also positively related to water column SRP concentration for NO<sub>3</sub><sup>-</sup> ( $r^2 = 0.46$ ,  $P = 0.05$ ) and NH<sub>4</sub><sup>+</sup> ( $r^2 = 0.60$ ,  $P = 0.02$ ).

#### Experiment 2: molecular analysis of enriched cellulose biofilms

CR on cellulose sponge in autumn 2006 in State Creek indicated N and P co-limitation (as NO<sub>3</sub><sup>-</sup>; Fig. 5A). Our cloning-DGGE-sequencing approach with bacterial primers detected 2–4 bands per

treatment, indicating that each biofilm contained only a few dominant taxa. Phylogenetic analysis of dominant bacterial 16S rRNA genes suggested differences in the dominant taxa based on which treatment was applied (Fig. 5B). Control, NH<sub>4</sub><sup>+</sup> and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3-</sup> had sequences most similar to Cytophaga-Flavobacteria, the Comamonadaceae family of Beta-Proteobacteria, Cyanobacteria, and a picoplankton chloroplast. In the NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, and NO<sub>3</sub><sup>-</sup> + PO<sub>4</sub><sup>3-</sup> treatments, we detected sequences most similar to Cytophaga-Flavobacteria, Gamma-Proteobacteria, and two organisms with sequences similar to a Beta-Proteobacteria cluster for which the family grouping is unresolved (Fig. 5B).

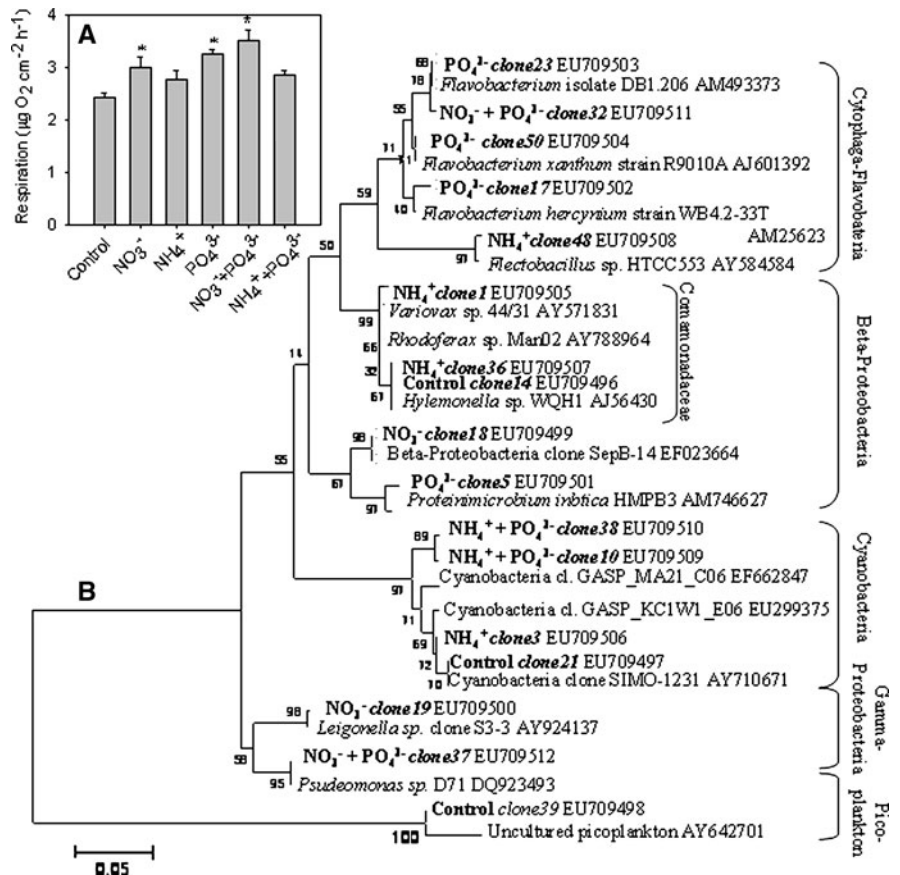
Cloning-DGGE-sequencing analyses with fungal primers showed only four taxa across all treatments, indicating even lower fungal diversity than bacterial diversity. Phylogenetic analysis of fungal sequences showed a high degree of similarity between two of the bands, one found in the control, NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup>, NH<sub>4</sub><sup>+</sup>, and NO<sub>3</sub><sup>-</sup> + PO<sub>4</sub><sup>3-</sup> treatments (Band 2), and the other in the PO<sub>4</sub><sup>3-</sup> and NH<sub>4</sub><sup>+</sup> + PO<sub>4</sub><sup>3-</sup> treatments (Band 1; Fig. 6). A BLAST search of Bands 1 and 2 sequences showed no matches in the NCBI database. Bands 3 and 4, found only in the NO<sub>3</sub><sup>-</sup> treatment, were similar to sequences obtained from cellulose buried in fertilizer-amended soil (NCBI Accession No. AY704760; Zhao et al., 2005) and wood-chip derived compost (NCBI Accession No. DQ900996; Fig. 6). We had two contaminant organisms isolated from our negative control, which were shown to most closely match the cosmopolitan Ascomycota fungi *Aureobasidium pullulans* and *Cladosporium* sp.

## Discussion

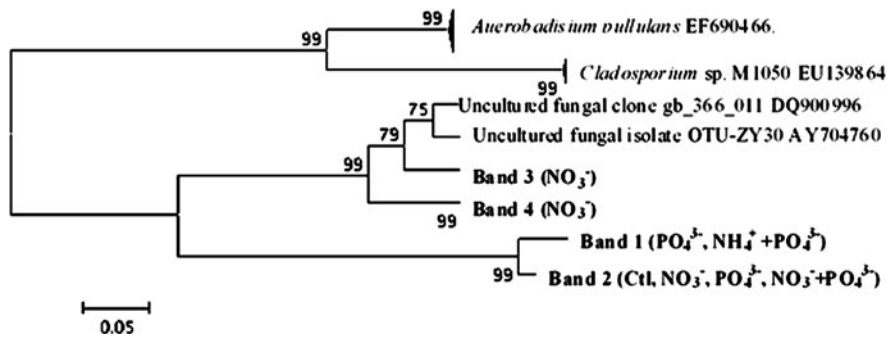
### Substratum type influences biofilm colonization

Our results suggest fundamental differences in biofilm structure and function on each substratum. Similar to previous studies (Tank & Dodds, 2003; Johnson et al., 2009), we found greater primary producer biomass on inorganic relative to organic substrata (Fig. 1). Autotrophic biofilm constituents likely out-compete heterotrophic constituents on inorganic substrata due to C fixation; heterotrophs were likely limited by a lack of organic C. In contrast, heterotrophs were likely more prevalent on cellulose

**Fig. 5** **A** Respiration on cellulose sponge amended with no nutrients (control), nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ),  $\text{NO}_3^- + \text{PO}_4^{3-}$ , and  $\text{NH}_4^+ + \text{PO}_4^{3-}$  in State Creek in autumn (11 Oct.–7 Nov. 2006). \* indicates significant nutrient limitation of  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{NO}_3^- + \text{PO}_4^{3-}$  treatments. **B** Phylogenetic tree of isolated PCR products amplified with bacterial primers representing the dominant taxa in each treatment. Bacteria were extracted from the same biofilms whose respiration rates are shown in **A**. Scale bar represents an estimated 5% divergence



**Fig. 6** Phylogenetic tree of isolated PCR products amplified with fungal primers representing the dominant taxa in each treatment. DNA was extracted from cellulose amended with no nutrients (control), nitrate ( $\text{NO}_3^-$ ), phosphate ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ),  $\text{NO}_3^- + \text{PO}_4^{3-}$ , and  $\text{NH}_4^+ + \text{PO}_4^{3-}$  incubated



due to ample labile C. Using the same substrata, Johnson et al. (2009) found similar results from NDS incubated in 72 streams throughout North America. Mean CR on cellulose was about equal between their study and ours ( $\sim 5.3 \mu\text{gO}_2 \text{ cm}^{-2} \text{ h}^{-1}$ ), suggesting patterns of biofilm colonization on inorganic and

for 28 days in State Creek starting November 7, 2006. Scale bar represents an estimated 5% divergence. *Aerobasidium pullulans* and *Cladosporium* sp. represent contaminants in fungal primers

organic substrata are consistent when considered across a wide geographic (Johnson et al., 2009) and temporal scales (this study).

Differences in functional properties of biofilms on organic and inorganic substrata have implications for managing stream ecosystems in the context of

nutrient enrichment. For example, epilithic (i.e., inorganic substrata) and epixylic (i.e., wood) biofilm communities have different metabolism, nutrient uptake rates, and patterns of nutrient limitation (Tank & Dodds, 2003). In these study streams, we have documented significant differences in metabolism and  $\text{NO}_3^-$  uptake among multiple biofilm substrata (e.g., wood, sand, and rocks; Hoellein et al., 2009). While the majority of previous NDS studies have used only inorganic substrata to assign the nutrient limitation status of the entire stream ecosystem (but see Tank & Dodds, 2003, Johnson et al., 2009), biofilms which control nutrient cycling in streams exhibit high spatial and temporal variability (i.e., hot-spots and hot moments; McClain et al., 2003). A more accurate representation of nutrient limitation may require enhanced spatial (i.e., multiple substrata) and temporal (i.e., seasonal) replication (Francoeur et al., 1999, Wold & Hershey, 1999).

Nutrient limitation on inorganic substrata was infrequent and influenced by seasonality

Nutrient limitation patterns for biofilms on inorganic substrata showed unexpectedly few cases of nutrient limitation, even though ambient nutrient concentrations were very low. Lack of nutrient limitation for primary producer biomass on inorganic substrata in oligotrophic streams in the same geographic region was also reported by Wold & Hershey (1999). They found a mixture of N, P, and N + P co-limitation, but recorded no nutrient limitation in 19 out of 72 cases (26%), by measuring chl *a* accrual on an inorganic substratum over a similar range of sampling dates. Biomass of primary producers was less frequently limited in our study (66% of cases show no limitation), demonstrating that streams in close proximity, or in the same biome, may not necessarily show similar nutrient limitation status, even where there are minimal human impacts on watershed conditions (i.e., “pristine” conditions). In addition, our collective data indicate that under oligotrophic conditions, nutrient limitation of biofilms on inorganic substrata should not be assumed, but the response of biofilms on inorganic substrata to nutrient enrichment is dependent on additional environmental factors.

The strongest relationship between physiochemical factors and biofilm NRR on fritted glass was in the summer, water temperature and SRP concentrations

were highest (Fig. 4). While we cannot distinguish the individual influences of water column SRP versus temperature (i.e., they were both changing with season), both factors could influence the response of biofilms to nutrient enrichment (Francoeur et al., 1999). Water column SRP concentrations were low (i.e.,  $<10 \mu\text{g l}^{-1}$ ), so biofilm organisms would likely be sensitive to changes in P. Biofilms on inorganic substrata assimilate water column nutrients (as  $^{15}\text{N}$ ) even when growing on NDS (von Schiller et al., 2007). In addition, water temperatures across all NDS deployments spanned a broader range than has been considered in previous NDS studies, 1–15°C (but see Francoeur et al., 1999; Wold & Hershey, 1999), suggesting temperature can regulate biofilm response to nutrient enrichment when considered across a large gradient.

Light availability is a primary controlling factor for growth of autotrophic constituents of lotic biofilms (Mosisch et al., 2001), so we expected the greatest response primary producer response to nutrients in spring (before leaf-out) and/or autumn (after leaf-fall). Canopy cover was an important driver of primary producer biomass on control substrata; however, the greatest response to nutrient enrichment was in the summer, when canopy cover was greatest. This contradicts other studies, which have shown nutrient enrichment has a greater effect on autotrophic biofilms with high relative to low light conditions (Tank & Dodds, 2003; Johnson et al., 2009). These previous studies included streams across multiple biomes, however, not seasonality within forested, headwater streams. It is likely that light availability at our study sites was low throughout the year relative to sites considered to be “open canopy” (e.g., streams in grasslands or deserts), which elevated the importance of temperature and water column SRP in driving response of primary producer biomass following nutrient enrichment.

Biofilm growth on NDS was inhibited in several cases, especially for respiration of biofilms on inorganic substrata, a phenomenon that has been found elsewhere (Tank & Dodds, 2003; Johnson et al., 2009). It is critical for managers and researchers to document when eutrophication may cause biofilm growth inhibition rather than stimulation. As in other studies, we cannot directly explain inhibition patterns with these data, but report it here and concur that it represents a potentially significant avenue of research with NDS.

### Nutrient limitation on organic substrata was frequent, not influenced by seasonality

In contrast to nutrient limitation on fritted glass, we found consistent patterns of nutrient limitation for respiration on cellulose sponge among seasons and streams. The results were consistent with seasonal patterns of whole-stream nutrient uptake (via short-term enrichments) measured in the same streams (Hoellein et al., 2007). Our previous work showed spring was the period of highest nutrient demand and one stream, State Creek, had the highest uptake rates. Patterns of nutrient limitation on cellulose matched these results: each stream was nutrient limited in spring, and State Creek was always nutrient limited. Because the nutrient limitation status of biofilms on organic substrata corresponded to the spatial and temporal patterns of whole-stream uptake, which integrates the all biofilm activity, biofilms on organic substrata were likely a significant portion of whole-stream nutrient demand across dates.

Contrary to our expectations, there were no significant relationships between environmental drivers and biofilm response to nutrient addition (i.e., NRR) for cellulose biofilms. These results contrast with those for inorganic substrata, and stem from the ability of biofilms on organic substrata to obtain C and/or nutrients from the substratum itself, while biofilms on inorganic surfaces are more dependent on water column supplies. Previous work in these streams supports this pattern; seasonal patterns of metabolism and  $\text{NO}_3^-$  uptake showed biofilm metabolism on rock surfaces was more strongly linked to changes in water column N concentration than metabolism on organic surfaces (Hoellein et al., 2009). Similarly, Tank & Dodds (2003) found the water column dissolved inorganic N:SRP ratio was related to the NRR following N enrichment for primary producer biomass, but was unrelated to fungal biomass. Therefore, biofilm growth on organic substrata may be less sensitive to water column conditions compared to biofilms on inorganic substrata due to facultative exploitation of nutrients from organic sources.

### No biofilm preference for $\text{NH}_4^+$ versus $\text{NO}_3^-$

Previous NDS studies have rarely employed a direct comparison of N-limitation by  $\text{NO}_3^-$  versus  $\text{NH}_4^+$

(but see von Schiller et al., 2007). Theoretically,  $\text{NH}_4^+$  is the preferred inorganic N-species relative to  $\text{NO}_3^-$  because it is the form required within the cell (Dortch, 1990) and requires no additional energy to bring across the cell wall. Among the few studies to test inorganic N preference,  $\text{NH}_4^+$  relative to  $\text{NO}_3^-$  preference has been shown for marine phytoplankton (Dortch, 1990), and stream periphyton (von Schiller et al., 2007). If  $\text{NH}_4^+$  is preferred, we would have expected to N-limitation from  $\text{NH}_4^+$  or  $\text{NH}_4^+ + \text{PO}_4^{3-}$  enrichments exclusively, and not via  $\text{NO}_3^-$  enrichment. However, our results do not support a  $\text{NH}_4^+$  versus  $\text{NO}_3^-$  biofilm preference on inorganic or organic substrata, because each N type, alone or in combination with P, was limiting to biofilm respiration and primary producer biomass multiple times. In fact, in one case we recorded N-limitation from  $\text{NO}_3^- + \text{PO}_4^{3-}$  and  $\text{NH}_4^+ + \text{PO}_4^{3-}$  treatments simultaneously. Future NDS measurements would benefit from incorporating multiple inorganic N-species when possible, because either can be limiting to biota, even in the same stream at the same time. In addition, to more fully develop our understanding of N-species preference for stream biofilms, we recommend including organic N treatments.

### Microbial communities show low diversity on nutrient-amended cellulose

Unexpectedly, the diversity of bacterial and fungal ribotypes observed on nutrient-amended cellulose sponge was at the low end of the range found for leaf litter and sediments in streams using DGGE (Battin et al., 2001; Brummer et al., 2003; Das et al., 2007). Using identical primers, protocols, and equipment, however, we found only slightly higher numbers of microbial taxa isolated from leaf litter in a North Carolina stream (Kominoski et al., 2009). The low diversity on the NDS could reflect the structural homogeneity of the cellulose sponge relative to the chemically heterogeneous C polymers present in leaf litter and sediments, which may support a more diverse microbial community. Low NDS microbial diversity could also be the result of shorter NDS incubation times relative to leaf litter in Kominoski et al. (2009). Bärlocher et al. (2006) used DGGE and conidia counts to show lower number of fungal taxa on wood compared to leaf litter, and our data suggest a similar pattern for leaves versus cellulose. We

acknowledge that the low diversity may reflect a methodological artifact of selecting for the dominant taxa using the modified DGGE clone screening technique (Green et al., 2007). Using the typical clone library approach, additional replicates, or other molecular techniques may have included a greater diversity of less dominant taxa. Finally, we acknowledge that DGGE bands may contain >1 taxon. Despite these considerations, biologically meaningful patterns can be deduced from these data, and results are promising for future research that pairs NDS and microbial taxa identification to explore the relationship between nutrient limitation and the stream biofilm structure and function.

The bacterial 16s rRNA sequences detected in the cellulose biofilms represent common constituents of aquatic microbial communities. Cytophaga-Flavobacteria are chemo-organotrophic bacteria which degrade polymers such as chitin and cellulose (Kirchman, 2002), and some are low-temperature adapted (Battin et al., 2001). Beta-Proteobacteria are prevalent early in biofilm formation (Manz et al., 1999), degrading the same or lower molecular weight C substrates as Cytophaga-Flavobacteria (Cottrell & Kirchman, 2000). Gamma-Proteobacteria have been found to be less abundant in streams than the two former groups (Olapade & Leff, 2005), but respond to nutrient enrichment (Pinhassi & Berman, 2003). Cyanobacteria have been described via DGGE and cloning, typically in water bodies with low N/P ratios, and concurrent with Beta-Proteobacteria and Cytophaga-Flavobacteria (Roeselers et al., 2007). Collectively, these taxa decompose high molecular weight C compounds, inhabit eutrophic environments, and are adapted to cold conditions, so they are well-adapted to the habitat provided by the high nutrient, cellulose substratum in autumn in northern Michigan.

NDS deployed in State Creek in autumn 2006 had significantly higher respiration rates for the  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{NO}_3^- + \text{PO}_4^{3-}$  treatments, and resulted in a phylogenetically distinct set of dominant bacterial taxa among treatments, suggesting a potential link among nutrient enrichment, microbial community structure, and ecosystem function. For example, growth of Cytophaga-Flavobacteria similar to *Flavobacterium* were dominant taxa colonizing cellulose with  $\text{PO}_4^{3-}$  and  $\text{NO}_3^- + \text{PO}_4^{3-}$  enrichments, but were not found to be dominant on the control,  $\text{NH}_4^+$  or  $\text{NH}_4^+ + \text{PO}_4^{3-}$  enriched biofilms. Olapade & Leff

(2005) and Rubin & Leff (2007) also linked increases in Cytophaga-Flavobacteria abundance with seasonal peaks in  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  concentrations in the Mahoning River, Ohio, and concluded this group required high nutrient availability to break down high molecular weight C compounds. The appearance of those taxa on the  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  amendments may have increased respiration rates. In general, empirical links among resource availability, microbial community composition, and ecosystem function in streams has received the most attention for leaf litter decomposition rates (Kominoski et al., 2010), and less attention has been directed toward experimental manipulations of nutrient availability. Our data suggest that NDS approaches will be valuable in advancing the understanding of how nutrient enrichment influences the relationship between microbial community structure and metabolism in stream biofilms.

Kominoski et al. (2009) recently used a DGGE approach to show that bacterial, rather than fungal diversity more strongly explained variation in leaf litter decomposition rates across a range of leaf species. Our results concur, although strong conclusions regarding fungal diversity are difficult to generate from the low number of taxa. In addition, the number of studies using DGGE and sequencing to analyze fungal communities on stream substrata other than leaf litter is low (Nikolcheva & Bärlocher, 2005; Das et al., 2007). We found two sequences in the NCBI database similar to ours, isolated from bio-fertilizer-amended soils (Zhao et al., 2005) and from woodchip-derived compost. This study represents a relatively early entry of fungal ITS sequences from cellulolytic stream fungi into the NCBI database. More research is needed to supply fungal sequences in the database, and resolve the relationship between nutrient enrichment and fungal diversity on organic substrata besides leaves.

## Conclusions

Dodds (2006) highlighted the importance of considering nutrient limitation of primary production and decomposition in streams, as the influence of the former has received more attention than the latter. Recent work has documented variability in nutrient limitation status on organic and inorganic substrata on streams in different biomes (Tank & Dodds, 2003) and different watershed land-use (von Schiller et al.,

2007; Johnson et al., 2009). Our study demonstrates that seasonality is also a critical factor influencing biofilm nutrient limitation among different substrata. In addition, enrichment by different species of inorganic N can affect biofilm activity and biomass. Finally, changes in biofilm function following nutrient enrichment may be linked with changes in the dominant bacterial taxa. Microbial diversity of dominant taxa on artificial substrata was lower than natural surfaces, however, and more analyses are needed which employ a diversity of both molecular and microscope techniques to further explore the relationships suggested here. Documenting the influence of seasonality on nutrient limitation of microbial biofilms is critical to refine management strategies to maintaining stream ecosystem health across varying levels of nutrient availability.

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