Response of heterotrophic stream biofilm communities to a gradient of resources

D. J. Van Horn1,*, R. L. Sinsabaugh1, C. D. Takacs-Vesbach1, K. R. Mitchell1,2, C. N. Dahm1

1Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131, USA
2Present address: Department of Microbiology & Immunology, University of British Columbia Life Sciences Centre, Vancouver BC V6T 1Z3, Canada

ABSTRACT: The metabolism and biogeochemical cycles of aquatic ecosystems are largely mediated by microbial communities, with biofilm assemblages dominating in stream ecosystems. To determine the effects of the availability of resources on the structure and function of heterotrophic stream biofilms, we created an enrichment gradient by amending darkened stream channel mesocosms with a stoichiometrically balanced solution of sucrose, NO3− and PO43−. A total of 1902 bacterial partial 16S rRNA gene sequences yielded 293 unique operational taxonomic units (OTUs, 97% sequence similarity). Significant differences (p < 0.005) were detected between communities from all treatments with the exception of the 2 highest enrichment levels, with increasing enrichment resulting in progressive community divergence and declining diversity. Both the productivity and function of the biofilm community increased exponentially with enrichment, with exponents of 1.5 for areal mass, 2.3 for live-cell density, and 2.5 to 3.5 for the activities of 5 extracellular enzymes. The nonlinear increase in functional capacity suggests that heterotrophic biofilms are highly responsive to the availability of resources, probably as a result of the physical structures and synergistic social interactions found in biofilm assemblages.

KEY WORDS: Heterotrophic stream biofilm · Resource gradient · Extracellular enzymes · 16S rRNA gene sequences

INTRODUCTION

The metabolism and biogeochemical cycles of aquatic ecosystems are largely mediated by microbial communities. Aquatic microbial communities occur in 2 primary forms: (1) planktonic assemblages that develop in the water columns of marine, lentic and large river environments, and (2) attached biofilm communities whose contributions to metabolism in the ecosystem are most prominent in small to mid-sized streams, wetlands and shallow lakes. While plankton and biofilm communities share similar biogeochemical and organic carbon processing functions, they have markedly different physical structures and biotic interactions that may lead to differing responses to changes in the supply of resources.

Planktonic microbial communities lack self-imposed physical structure. Metabolism is regulated by top-down (predator–prey) and bottom-up (resource availability) interactions. Bottom-up effects are dominant in oligotrophic systems and top-down interactions are most important in eutrophic environments (Dufour & Torreton 1996, Gasol et al. 2002, Vargas et al. 2007, Thelaus et al. 2008). Within planktonic communities there is little evidence for cooperative or synergistic interactions among populations. Predator–prey interactions create a microbial loop that either packages carbon and nutrients for higher trophic levels or returns dissolved carbon and nutrients to support bacterial production.

In contrast to planktonic microbial communities, attached microbial populations form complex structured associations in biofilms. When planktonic microbial cells adhere to solid surfaces, signal cascades alter gene expression and initiate the formation of a dense layer of extracellular polymeric substances (Watnick & Kolter 2000, Beloin & Ghigo 2005) that shield biofilm organisms from predators and insulate
inhabitants from external variables such as pH, temperature, ultraviolet light, desiccation, and toxic or antimicrobial substances (Webb et al. 2003, Hall-Stoodley et al. 2004). As biofilms thicken, physical and chemical gradients form internally, promoting cooperative metabolic interaction within and between populations (Costerton et al. 1995, Davey & O’Toole 2000). Populations interact through multiple intercellular communication mechanisms, including quorum sensing (Hense et al. 2007), programmed cell death within populations (Webb et al. 2003), and lateral gene transfer within and among species (Watnick & Kolter 2000, Parsek & Fuqua 2004).

The substantial differences between planktonic and biofilm associations may influence the response of these communities to an altered supply of resources. Because they are simpler to manipulate, planktonic communities have been the focus of most studies that examine the composition and metabolism of microbial communities in relation to the availability of resources. Meta-analyses of cross-site eutrophication gradients show that planktonic primary production increases linearly with nutrient enrichment, stimulating a linear, but less than 1:1, increase in bacterioplankton production (Cole et al. 1988, Thelaus et al. 2008). Bacterial biomass, however, increases more slowly than production as a result of increased predation (Thelaus et al. 2008). Similar patterns have been observed using experimental mesocosms through the manipulation of primary production (Hobbie & Cole 1984, Oviatt et al. 1986) and direct additions of C, N and P (Joint et al. 2002, Smith & Prairie 2004, Jansson et al. 2006).

In contrast, studies on the effects of the supply of resources on the structure and function of biofilm communities are limited. Nutrient or carbon enrichment from either anthropogenic sources (Brümmer et al. 2003, Kobayashi et al. 2009) or experimental manipulations (Olapade & Leff 2005, 2006) have been shown to alter the composition of the biofilm community and the abundance of bacteria from different phyla, sub-phyla, genera and species. Experimental manipulations show that the greatest changes occur in response to additions of labile carbon, while the addition of complex carbon stimulates or suppresses various members of the microbial community (Olapade & Leff 2005, 2006). A whole-stream addition of nitrogen and phosphorus significantly affected microbial function (Stelzer et al. 2003). Respiration rates increased for a variety of carbon substrates, with the greater increases associated with low-nutrient substrates. Several experimental single-level enrichments in microcosms suggest that the simultaneous addition of C, N and P alters community composition (Ylla et al. 2009) and—even at low levels—leads to substantial, and possibly non-linear, changes in biofilm production and function (Mohamed et al. 1998, Chenier et al. 2003, 2006). While the studies mentioned above have investigated the effects of adding carbon or nutrients, or single-level carbon and nutrients, on various aspects of stream biofilms, to our knowledge no study has investigated the effects of an enrichment gradient on the structure and function of a biofilm community.

In this study, we used stream mesocosms to measure the effects of a gradient of resources—that included carbon, nitrogen and phosphorus—on heterotrophic biofilm productivity, potential extracellular enzyme activity (EEA), and community diversity and structure. We compared the magnitude and linearity of these responses in relation to the supply of resources, and contrasted these responses to those reported for planktonic microbial communities.

**MATERIALS AND METHODS**

**General experimental design.** Heterotrophic microbial biofilms were established in complete darkness in 15 experimental stream channel mesocosms (Singer et al. 2006). The channels (depth/width/length: 0.02/0.1/3.0 m) were lined with removable unglazed ceramic tiles, supplied with stream water to ensure colonization by ambient microbial populations, and continually enriched with a stoichiometrically balanced (C:N:P ratio 106:16:1) solution of dissolved organic carbon (DOC) in the form of sucrose, NO₃⁻ (NaNO₃), and PO₄³⁻ (KH₂PO₄) (Fig. 1). Labile substrates were chosen to ensure that a productivity gradient was established. Water was diverted into the mesocosms from an intermittent stream located in Bear Canyon in the foothills of the Sandia Mountains near Albuquerque, New Mexico. The stream water was filtered (10 µm filter) prior to delivery to a header tank that distributed water at a rate of 0.03 l s⁻¹ per mesocosm, generating a nominal flow velocity of ~2 cm s⁻¹ within the channels. Enrichment treatments were multiples of the ambient DOC concentration (~1.5 mg l⁻¹). Treatments included no enrichment in control channels, and 2×, 4×, 8× and 10× increases in ambient DOC concentration with supplemental N (NO₃⁻, ambient concentration = 0.002 mg l⁻¹ NO₃⁻-N) and P (PO₄³⁻, ambient concentration = 0.002 mg l⁻¹ PO₄³⁻) added to preserve a C:N:P ratio of ~106:16:1. Nutrient solutions were metered into channels behind baffles to ensure even mixing. Three replicate channels were used for each level of enrichment. After 3 wk of growth, from May 8 to May 28, 2008—the time necessary to produce a mature biofilm—colonized tiles were collected for biofilm analyses. Three replicate tiles were collected for analysis for each biofilm production/function measurement from the same location in each channel to facilitate between-
channel comparisons. A single tile was analyzed from each channel for 16S rRNA gene sequences.

**Biofilm productivity and physical structure.** Biofilm mass (dry mass, DM), and ash-free dry mass, AFDM) was measured by scraping tiles with a metal spatula and depositing biofilm material onto ashed, weighed glass-fiber filters placed in aluminum pans. Filters were dried at 80°C, reweighed to calculate DM, and ashed at 500°C for 3 h. Ashed filters were reweighed and AFDM was calculated as the difference between dry mass and ash mass.

The relative abundance of live and dead cell biomass was assessed using a BacLight™ kit (Invitrogen). The stains were checked for linearity of fluorescence over the biofilm cell densities to be analyzed using serial dilutions of a high-biomass sample. Sample blanks were also performed using 50 mM bicarbonate buffer. Samples were homogenized in a 50 mM bicarbonate buffer (pH 8), eight 250 µl replicates for each sample were pipetted into black 96-well microplates, and 6 µl of an equal mixture of a 1:10 dilution of the stains were added to the microplate wells. Samples were incubated at room temperature in the dark for ~15 min and then read on an fmax Fluorescence Microplate Reader (Molecular Devices) set to an excitation wavelength of 485 nm and an emission wavelength of 538 nm for the SYTO® 9 stain, and an excitation wavelength of 485 nm and an emission wavelength of 591 nm for the propidium iodide stain. Results were normalized by the area of the tile sampled and were corrected for dilution where appropriate. The microplate method described above has been validated for measuring live and dead cell abundance in single-species cultures (Alakomi et al. 2005). However, when used in a complex community, or to compare communities dominated by organisms with different cell morphologies, as in the present study, the values of fluorescence represent the relative abundance of live and dead cell biomass rather than the absolute abundance of cells.

The physical structure of biofilms was assessed using confocal microscopy. Briefly, a single biofilm-colonized tile from each channel was placed in a plastic tray while still submerged in the experimental stream channel, and transported on ice to the Keck Confocal Laboratory at the University of New Mexico. Samples were stained with the live/dead stain described above and imaged using a 5× objective on a LSM 510 confocal microscope (Carl Zeiss).

**Biofilm function.** Biofilm function was assessed by measuring the potential activity of 5 hydrolytic extracellular enzymes: α-glucosidase (AG), β-glucosidase (BG), N-acetylglucosaminidase (NAG), alkaline phosphatase (AP) and leucine aminopeptidase (LAP). Potential activities were measured using methylumbelliferyl-linked substrates following protocols similar to those described by Sinsabaugh et al. (1997). Biofilm was homogenized in 50 mM bicarbonate buffer (pH 8). Aliquots (each 200 µl) of biofilm homogenate and 50 µl of 200 µM substrate were added to black, 96-well microplates with 16 replicate wells per sample. The microplates were incubated in the dark at room temperature. Each plate contained reference standards, substrate controls, and sample controls. Fluorescence was measured periodically for up to 19 h using an fmax Fluorescence Microplate Reader set to an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The fluorescence results were checked for linearity over the incubation period, and activities were calculated as nmol substrate converted per hour for each cm² of tile (nmol h⁻¹ cm⁻²).

**Biofilm community structure and diversity.** Samples for analysis of the 16S rRNA gene sequence were
stored in sucrose lysis buffer (SLB) at −80°C until extraction with the cetyltrimethylammonium bromide (CTAB) method as described by Mitchell & Takacs-Vesbach (2008). Bacterial 16S rRNA gene sequences were amplified using the bacteria-specific forward primer 8F 5′-AGA GTT TGA TCC TGG CTC AG-3′ and the reverse primer 1492R 5′-GTT TAC CTT GTT ACG ACT T-3′ (Lane 1991) in triplicate 50 µl reactions containing 5 µl 10× buffer (Promega Buffer B with 1.5 mM MgCl2), 12.5 mM each dNTP (BioLine USA), 20 pmol each of 8F and 1492R primers, and 2.5 U Taq polymerase (Promega). The PCR thermal cycling profile was 10 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, 90 s at 72°C; 7 min at 72°C (ABI GeneAmp 2700, Applied Biosystems). Triplicate 16S rRNA gene amplifications were pooled and gel-purified using a DNA Purification Kit (MoBio Laboratories), and cloned using a TOPO TA Cloning Kit (Invitrogen). For each library, 192 clones were sequenced using high-throughput Sanger sequencing (ABI 3730 Capillary Sequencer, Applied Biosystems), half with vector specific primers (M13) and half with 8F primers. Sequences were deposited in GenBank under the accession numbers JF695046 to JF696947.

Data analysis. Differences among enrichment treatments for biomass, enzyme activities, and live/dead cell biomass were assessed using 1-way analysis of variance (1-way ANOVA) and Bonferroni multiple comparisons on log-transformed data using SAS® software (Version 9.2, Copyright SAS Institute). Standardized Major Axis Tests & Routines (SMATR, Falster et al. 2006, Warton et al. 2006) was used to calculate scaling exponents (b) and 95% confidence intervals (CI) for biomass, enzyme activities, and live/dead cell biomass using ordinary least squares regression of log-transformed data. Lines were fit to C:P (BG:AP) and C:N (BG:[LAP+NAG]) enzyme ratios using the Standard Major Axis method (SMA) and to determine significant differences in the slopes of these lines.

Biofilm community 16S rRNA gene sequence data were checked for quality using CodonCode Aligner. High-quality sequences (containing >400 bases with PHRED scores >20) greater than 500 bp were exported to Greengenes (http://greengenes.lbl.gov) for alignment (NAST Alignment Tool; DeSantis et al. 2006) and chimera checking (Bellerophon Chimera Check Tool; Huber et al. 2004). The ARB software package was used to filter the sequences to a uniform length and to create a distance matrix of the aligned and filtered sequences (Ludwig et al. 2004). The distance matrix was analyzed in mothur (Schloss et al. 2009) to cluster sequences into OTUs, to generate rarefaction curves using a 97% DNA sequence similarity cutoff, to calculate the Chao1 estimate of richness, and to determine the abundance of each OTU in each sample. Mothur was also used to assign taxonomic classifications to each OTU using bacterial reference sequences from the SILVA rRNA database project.

The phylogeny of the bacterial 16S rRNA genes from the enrichment gradient was analyzed using UniFrac (Lozupone & Knight 2005, Lozupone et al. 2006). Briefly, all aligned, high-quality sequences were added to a backbone phylogenetic tree of ~6500 bacterial 16S rRNA gene sequences (Hugenholtz 2002) using the parsimony add function in ARB (Ludwig et al. 2004). This tree was imported into UniFrac to calculate the UniFrac metric as described by Lozupone & Knight (2005). Unweighted UniFrac hypothesis testing was performed to test whether the communities from the 5 treatments had the same structure. A principal coordinate analysis (PCA) was performed in UniFrac (Lozupone et al. 2006).

**RESULTS**

Physical structure of the biofilm

Mean AFDM values in the control, 2× and 4× enrichments ranged from ~0.1 to 0.25 mg cm−2 while values in the 8× and 10× enrichments ranged from ~1.0 to 1.25 mg cm−2 (Fig. 2). AFDM values for the control and 2× enrichment were similar and not statistically different (p > 0.05). The values for the 8× and 10× enrichments were also similar to each other. The areal density of AFDM and DM responded in a roughly linear fashion to the resource gradient, with 23- and 18-fold increases (b ± 95% CI; AFDM = 1.48 ± 0.28, DM = 1.31 ± 0.29), respectively, over the 10-fold enrichment gradient (Fig. 3).

Confocal laser microscopy showed that biofilm from the control treatment was a dense layer of bacterial cells with minimal vertical development. Images from successive enrichments showed increasing vertical development driven by increasing abundance of filamentous growth forms interspersed with cocci and rod-shaped cells (images not shown). As a result, the areal density of AFDM and DM increased much more than AFDM in response to resource enrichment, with increments of 10-, 53-, 213- and-132 fold for AFDM, in the 2×, 4×, 8× and 10× enrichments, respectively (b ± 95% CI; abundance: 2.25 ± 0.23, AFDM: 2.05 ± 0.18) (Fig. 3). The live and dead cell fluorescence values for the control, 2× and 4× treatments were significantly different from one another and from the 8× and 10× treatments, which were not statistically different (Fig. 2). One of the 15 channels, channel 9 — a 4× treatment — was a consistent outlier and was excluded from the analyses of biofilm physical structure and function.
Biofilm function

As biofilm biomass increased in response to enrichment, EEA increased exponentially (Fig. 3). In relative terms, EEA increased in the order AG, NAG, BG, LAP and AP (Fig. 4). For AG, activity in the control to 10× enrichment treatments ranged from 0.004 to 8.0 nmol h⁻¹ cm⁻², respectively; for AP the corresponding range was 0.14 to 47 nmol h⁻¹ cm⁻². Response ratios for the 10× enrichment relative to the control treatment increased in approximately the reverse order relative to the absolute EEA, with values of 350, 500, 1100, 2100 and 7900 for AP, LAP, BG, AG and NAG, respectively (Fig. 3). Exponential scaling exponents ($b$ ± 95% CI) were $2.42 \pm 0.26$, $2.48 \pm 0.35$, $2.76 \pm 0.40$, $3.01 \pm 0.43$ and $3.46 \pm 0.52$ for AP, LAP, BG, AG and NAG, respectively (Fig. 3). Only 1 enzyme, LAP, reached a plateau at the 8× enrichment, with a slight decrease in activity found in the 10× enrichment (Fig. 4). For each enzyme, activity in the control treatment was significantly different from that in enrichment treatments, while activities in the 8× and 10× enrichment treatments were not significantly different (Fig. 4).

Across the enrichment gradient, ratios of the activities of C-, N- and P-acquiring enzymes, represented as $B:(LAP+NAG):AP$, remained consistent at ~1:1:1 (Fig. 5). The standard major axis analysis of $ln(BG)$ vs. $ln(LAP+NAG)$, and $ln(BG)$ vs. $ln(AP)$ across the enrichment gradient revealed significantly ($p < 0.02$) different slopes ($\pm$95% CI) of $1.09 \pm 0.05$ and $1.20 \pm 0.07$, respectively (Fig. 5).

Composition of the biofilm community

A total of 1902 high-quality partial 16S rRNA gene sequences were obtained (see Table S1 in the supplement at www.int-res.com/articles/suppl/a064p149_supp.pdf). These sequences were distributed across the resource gradient treatments with 356, 397, 398, 400 and 351 sequences from the control, 2×, 4×, 8× and 10× enrichment treatments, respectively. A total of 293 OTUs were identified at the species level (97% sequence similarity) with 168, 70, 69, 46 and 36 OTUs found in the control, 2×, 4×, 8× and 10× enrichment treatments, respectively. Rarefaction curves from the 4 enrichment treatments (97% similarity cut-off) approached a plateau, indicating comprehensive sampling of the 16S rRNA gene diversity amplified in these samples (Fig. 6). The rarefaction curve from the control treatment did not reach a plateau; however, the slope of this curve was decreasing — indicating that a substantial amount of the 16S RNA gene diversity was successfully described (Fig. 6). Chao 1 estimates of species richness indicated that bacterial diversity progressively decreased with enrichment: the control, 2×, 4×, 8× and 10× enrichment treatments had richness estimates (97% sequence similarity) of 429 (95% CI: 325 to 604), 136 (102 to 211), 105 (85 to 153), 96 (72 to 160) and 60 (47 to 100), respectively.

Representatives from 11 bacterial phyla and candidate divisions were found in the 1902 partial 16S rRNA gene sequences recovered. Of the 11 phyla detected, 9, 5, 2, 4 and 4 were represented in the control, 2×, 4×, 8× and 10× enrichment treatments, respectively.
An analysis of OTU overlap between treatments showed the least overlap between the control and 10× treatments (5 OTUs) and the most overlap between the 4× and 8× (30 OTUs) and the 8× and 10× (28 OTUs) treatments. In general, samples from treatments with increasingly divergent levels of enrichment shared a decreasing number of OTUs (Fig. 7). The unweighted UniFrac hypothesis testing analysis indicated that communities from the 5 treatments were significantly different from one another (p < 0.005), with the exception of the 8× and 10× treatments. PCA results show relatively tight and distinctive grouping of sequences from the control treatment, from the 2× and 4× enrichment treatments and a grouping that includes sequences from the 8× and 10× treatments (Fig. 8). Principal coordinate 1 explained 28% of the variation in the samples and appeared to be well correlated with the eutrophication enrichment level as the sample clusters increased in enrichment from the left to the right side of the plot (Fig. 8).

Classification of the partial 16S rRNA gene sequences (mothur, SILVA taxonomy) revealed taxonomic shifts in the most frequently found sequences from each treatment. Sequences that could be identified to genus level were the most evenly distributed in the control treatment, with the greatest percentage of sequences from the *Leptothrix* (*Betaproteobacteria*), *Rhodobacter* (*Alphaproteobacteria*) and *Flavobacterium* (*Bacteroidetes*) genera. Sequences from the 2× enrichment were most commonly found in the *Flectobacillus* (*Bacteroidetes*), *Arcicella* (*Bacteroidetes*) and *Leptothrix* (*Betaproteobacteria*) genera. The 4× enrichment was composed of *Rhodoferax* (*Betaproteobacteria*), *Arcicella* (*Bacteroidetes*) and *Flectobacillus* (*Bacteroidetes*) genera. The 8× and 10× enrichments were populated mainly with sequences from the *Aeromonas* (*Gammaproteobacteria*), *Janthinobacterium* (*Gamma-proteobacteria*), *Flectobacillus* (*Bacteroidetes*) and *Rhodoferax* (*Betaproteobacteria*) genera.

**DISCUSSION**

The bulk biomass of the heterotrophic biofilm community responded nonlinearly to enrichment with an exponent \(b\) of ~1.5 for areal mass. Bacterial produc-
Fig. 7. Relative abundance of bacterial 16S rRNA gene sequences ordered by taxonomy (SILVA classification) and enrichment level (multiples of ambient concentration of dissolved organic carbon, ~1.5 mg l\(^{-1}\)). Each row represents an OTU defined at 97% sequence similarity cutoff. The shading of each OTU bar represents the relative abundance of the sequences in each OTU for each enrichment treatment according to the key on the right-hand side. The phylum or candidate division of each OTU is indicated on the left and demarcated by thin black horizontal lines.
As biofilm bulk biomass increased in response to enrichment with resources, the activity of enzymes responsible for C, N and P acquisition increased more quickly (range of $b \sim 2.4$ to 3.5). These results are generally consistent with previous biofilm studies that used single-level enrichments. Modest 15 to 20% increases in C, N and P resulted in 3- to 200-fold increases ($b \sim 1.2$ to 3) in the functional processes of a mixed heterotrophic/autotrophic biofilm, including the utilization of carbon and nitrogen compounds (Chenier et al. 2003, 2006) and nitrification and denitrification rates (Chenier et al. 2003, 2006). In contrast to biofilms, net functional processes in planktonic microbial communities exhibit muted responses to increased resources. Several mesocosm studies have used additions of glucose to stimulate the removal of nitrogen by bacterioplankton; the net response ratios appear to be well below 1, suggesting that C enrichment impacted these functions minimally (Shiah & Ducklow 1995, Joint et al. 2002, Jansson et al. 2006). These results suggest that the differing magnitude of biomass/production responses of biofilm and planktonic communities to altered resources translates to the net functional responses of these communities. A given level of enrichment results in a smaller functional increase in planktonic communities, as measured by extracellular enzyme activities; however, the activity-to-production or activity-to-biomass ratios for the 2 types of community are similar (Sinsabaugh et al. 2010). This similarity is evident when turnover (% biomass d$^{-1}$) is compared across a wide variety of attached and planktonic bacterial communities. Biofilm turnover for environments—including decaying leaf litter, streams, mesocosm wetlands and tropical coastal lagoons—range from ~0.02 to 4000% d$^{-1}$ with a median value of ~48% d$^{-1}$ (see the summary table in Su et al. 2007, and see Thomaz & Esteves 1997a,b, Törnblom & Søndergaard 1999, Carr et al. 2005, Tao et al. 2007). If 2 extreme outliers are removed from the 23 measurements gathered from the available literature, mean and median biofilm turnovers are 48 and 30% d$^{-1}$ respectively. Bacterioplankton turnover for lakes and the open ocean ranges from ~3 to 180% d$^{-1}$ with mean and median values of 43 and 33% d$^{-1}$ respectively (Hyun et al. 1998, Torréton et al. 2002, Hyun & Kim 2003, Chen et al. 2005, Hyun & Yang 2005, Gao et al. 2007). Thus, while significant variability exists, median and mean turnover rates for the 2 types of community are very similar.

The responses of planktonic and biofilm communities to nutrient enrichment reflect their physical and community structures and the fate of their products. Bacterioplankton biomass increases moderately in response to increased resources, while production and predation increase very rapidly, with total predation increasing 1000-fold, and predation per unit of bacterio-

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**Fig. 8.** Principal coordinate analysis performed in UniFrac for the bacterial communities of the 15 individual enrichment channels (triplicates of control [1×], 2×, 4×, 8× and 10× multiples of the ambient concentration of dissolved organic carbon, ~1.5 mg l$^{-1}$). Analysis was performed using the UniFrac unweighted analysis option.
ial biomass increasing 100-fold, over a 2800-fold range in primary productivity (Thelaus et al. 2008). Thus, although bacterioplankton production rates are high, predation eliminates much of this biomass and is a primary factor controlling turnover rates. In biofilm communities, biomass increases moderately with increasing resources ($b \sim 1.5$). However, in our study, the activity of enzymes responsible for the depolymerization of senescent material increased much more rapidly than biomass, resulting in the rapid internal cycling of materials. While grazing can be important in regulating biofilms (Huws et al. 2005), attached bacteria produce chemical defenses that protect the communities from eukaryotic predation (Weitere et al. 2005, Matz et al. 2008) and modify their organization to create grazing-resistant structures (Wey et al. 2008). These results suggest that turnover in biofilms is a result of internal cycling supported by biofilm structural and community responses to enrichment. Structurally, increased biofilm thickness protects extracellular enzymes from being washed out of the system by downstream flow, and it allows biofilms to concentrate nutrients (Tsuchiya et al. 2009) and energy-yielding materials (Battin et al. 1999)—which are dispersed in planktonic communities. This protection probably also encourages the production of extracellular enzymes by quorum sensing (DeAngelis et al. 2008, Decho et al. 2010), diffusion sensing (Redfield 2002), or efficiency sensing (Hense et al. 2007), which occur when concentrations of small, energy-inexpensive signal molecules reach threshold levels, inducing the transcription of large, energy-expensive extracellular enzymes. The dramatic increase in EEA may also be linked to differential rates of enzyme expression related to changes in bacterial community composition (Martinez et al. 1996). Thus, although the present study lacked the resolution to differentiate between several possible underlying mechanisms, heterotrophic biofilm development is highly responsive to the availability of resources, effectively storing (Tsuchiya et al. 2009) and cycling available resources. Ultimate limits are probably imposed by physicochemical constraints such as shear strength and diffusion gradients (Battin et al. 2003, Besemer et al. 2007)—conditions likely to be found in biofilms in later successional stages rather than in the communities, described in the present study, that developed in 3 wk.

Comparing the responses of planktonic and heterotrophic biofilms to enrichment is potentially complicated by the autotrophic component present in planktonic systems that was absent from the current experiment. Our biofilm communities more closely resemble the heterotrophic microbial communities found in streams or microenvironments with low-light conditions. Numerous studies have compared the structure and function of heterotrophic biofilms to the mixed heterotrophic/autotrophic communities found in systems with adequate light to stimulate algal growth. Heterotrophic biofilms have lower bacterial abundances (Roman & Sabater 1999, Romaní et al. 2004, Ylla et al. 2009) and altered community compositions (Sinsabaugh & Linkins 1988, Ylla et al. 2009) when compared to biofilms grown in the light. Functional comparisons of these 2 types of biofilm reveal complex heterotrophic/autotrophic interactions. In general, heterotrophic biofilms are dependent on exogenous carbon sources: they respond rapidly and efficiently to simple carbon inputs, indicating a deficit of labile carbon (Romaní et al. 2004), and they have high ratios of hemicelluloses to cellulose consistent with a reliance on complex carbon sources when labile sources are unavailable (Roman & Sabater 1999). In contrast, bacteria in light-grown biofilms are more dependent on autotrophic production to supply metabolic substrates (Roman & Sabater 1999, Romaní et al. 2004). Thus, they are probably indirectly responsive to increases in nutrients via labile organic molecules leaked or secreted from elevated autotrophic production (Ylla et al. 2009). Differences in EEA between the 2 types of community are less clear. Some autotrophic communities have higher areal (Roman & Sabater 1999, 2000, Romaní et al. 2004, Ylla et al. 2009) and per cell extracellular enzyme activities (Roman & Sabater 1999); however, this does not always hold true (Sinsabaugh & Linkins 1988, Jones & Lock 1993). Thus, biofilms grown in the light under a similar enrichment gradient would probably respond somewhat differently than the heterotrophic communities studied here due to heterotrophic/autotrophic interactions. However, the general pattern of greater biofilm biomass, productivity, and functional responses—as compared to planktonic communities—is likely to hold for several reasons. First, phototrophic biofilms have been shown to respond positively to nutrient additions, given adequate light (Johnson et al. 2009). Second, in each comparison of light-versus-dark biofilms discussed above, the light-grown biofilms have had higher bacterial abundance and similar or higher activity. Third, in the very few cases in which paired heterotrophic and autotrophic biofilms have both received enrichment, bacteria from both types of community have responded similarly (Sobczak 1996, Ylla et al. 2009).

Unlike biomass and activity, which increased rapidly with the availability of resources, enrichment led to a decreased richness of bacterial OTUs. Numerous studies have shown that enrichment alters the general community structure of bacterial biofilm and planktonic communities (Lebaron et al. 2001, Schäfer et al. 2001, Chenier et al. 2003, 2006, Haukkia et al. 2006), but resolution has been limited. Declines in the richness of
bacterioplankton (Carlson et al. 2002, Bertoni et al. 2008), or in their evenness (Sipura et al. 2005), with enrichment have been observed in some planktonic studies, supporting the trend observed in the present study.

Enrichment also led to dramatic changes in the composition of bacterial communities. Moderate enrichment (2×) resulted in substantial shifts in the community composition, suggesting that stream biofilm communities from oligotrophic systems are highly responsive to enrichment. In the control treatment, *Bacteroidetes, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria* were present in approximately equal numbers, with *Verrucomicrobia* and *Actinobacteria* present at lower frequencies. These phyla are typical of those found in other stream biofilm communities (Brümmer et al. 2003, Besemer et al. 2007, Kobayashi et al. 2009, Ylla et al. 2009). In contrast, *Betaproteobacteria* and *Gammaproteobacteria* were the dominant phyla in the highest levels of enrichment, and OTUs related to *Verrucomicrobia* and *Actinobacteria* were absent. The shift to a *Gammaproteobacteria*-dominated community has been observed in other enrichment studies (Pinhassi & Berman 2003) and thus the observed decline in diversity may be attributable to the dominance of opportunistic weed-like species.

The dominant genera from each level of enrichment also changed dramatically; however, the majority of these genera are classified as chemoorganotrophs. Two of the dominant genera found in the control treatment, *Flavobacterium* and *Leptothrix*, are dominated by aerobic species, while the third genus, *Rhodobacter*, contains photoautotrophic, photoheterotrophic and chemotrophic organisms. Relatives of all 3 genera — *Flavobacterium* (Kobayashi et al. 2009), *Leptothrix* (Brümmer et al. 2003, Ylla et al. 2009) and *Rhodobacter* (Ylla et al. 2009) — have been found in other stream biofilms. Interestingly, *Rhodobacter* was found in another dark-grown biofilm (Ylla et al. 2009). The dominant genera in the 8× and 10× enrichments — *Aeromonas, Janthinobacterium* and *Arcicella* — have been found in other stream biofilms (Kobayashi et al. 2009); they consist primarily of chemoorganotrophs and are aerobes, with the exception of *Aeromonas*, which is a facultative anaerobe. The presence of this facultative anaerobe suggests that oxygen may have been limiting in the well-developed biofilms that resulted from enrichment.

Changes in the composition of the bacterial community have been related to enrichment in other aquatic systems. Ylla et al. (2009) found that the addition of glucose eliminated OTUs related to *Actinobacteria* from a community of heterotrophic bacteria, a pattern observed in our enrichment study. Kobayashi et al. (2009) used bacterial community profiling techniques to synoptically sample 2 rivers in Japan. Significant changes in the patterns of bacterial community composition were most strongly related to anthropogenic nitrogen inputs; however, insufficient sequencing prevented a determination of which bacterial genera were responsive to enrichment. A similar study of the Elbe River in Germany, and a highly polluted tributary, found that pollution primarily impacted the abundance rather than the presence/absence of bacterial genera (Brümmer et al. 2003). Relatives of the most common genera from the control and enrichment treatments described above have been found in biofilms from globally distributed locations. This ubiquity suggests that these genera are members of a cosmopolitan freshwater biofilm cluster.

In this study of heterotrophic bacterial biofilms, an enrichment gradient produced a productivity gradient; however, OTU richness declined at all levels of enrichment. This was surprising as richness was expected to increase with the number of physical and chemical niches that form as biofilms mature and thicken, a response that would have been consistent with the species-energy hypothesis (Allen et al. 2002). These findings suggest that, for heterotrophic bacterial communities, an increase in total available energy increases the absolute live-cell biomass as well as the live-to-dead cell ratio; however, the total number of viable bacterial populations decreases. Thus, the diversity of resources, rather than the total resources available, may be more important for structuring these communities. This is consistent with the results from 2 studies: (1) a laboratory culturing experiment which found that bacterial diversity had a unimodal relationship to productivity in heterogeneous — but not in homogeneous — environments (Kassen et al. 2000), and (2) a study involving a high-carbon soil environment in which resource heterogeneity was thought to drive diversity patterns (Zhou et al. 2002). Additionally, aquatic microbial communities have known sensitivities to varying carbon substrates. In stream biofilms, the abundance of cells from the domain bacteria and several phyla, sub-phyla, and species, shifted in response to the additions of glucose and to seasonal changes in available DOM (Olapade & Leff 2005, 2006), and the composition of the community changed rapidly in response to inoculation with a variety of simple and complex DOM substrates (Wu et al. 2009, Rodrigues et al. 2010). These studies also found that leaf leachate stimulated or suppressed various members of the microbial community, indicating differential utilization of the potential DOM pools (Olapade & Leff 2005, 2006). DOM utilization preferences have also been described for a variety of bacterial phyla, sub-phyla, and species in marine (Cottrell & Kirchman 2000), estuarine (Covert & Moran 2001) and stream biofilm (McNamara & Leff 2004, Lear et al. 2009) communities.
CONCLUSIONS

Stream biofilm communities play a significant role in processing nutrient and organic matter inputs to streams. As streams are enriched with nutrients and DOC, the community diversity and structure of these biofilms, as well as their ability to perform functional processes, undergo significant changes, with some similarities to those observed in planktonic communities. In both planktonic and biofilm communities, enrichment stimulates greater increases in productivity than in biomass; however, the magnitude of this response was much larger in the heterotrophic biofilms in our study. In planktonic communities, increased production is transferred up the food web where it stimulates secondary production and respiration, with modest increases in planktonic biomass. In biofilms, the products of nutrient enrichment (e.g., cells, enzymes, extracellular polymers) tend to accumulate, initiating a structural and social positive feedback that increases biomass and potential EEA. This positive feedback is eventually truncated by structural failures (sloughing) that export material downstream, or by invertebrate grazing. Further study of the effects of enrichment on biofilms using a complex carbon source, as opposed to the readily labile carbon source used here, would provide additional insights into productivity–diversity relationships as a more diverse community may be necessary to break down various fractions of a more complex carbon pool.

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LITERATURE CITED

► Decho AW, Norman RS, Visscher PT (2010) Quorum sensing
flow conditions and carbon concentrations. Bioresour Technol 101:6888–6894
Thomaz SM, Esteves FA (1997a) Bacterial dynamics in periphyton from different regions of a tropical coastal lagoon. Arch Hydrobiol 139:495–507
Thomaz SM, Esteves FD (1997b) Secondary productivity (1H-leucine and 3H-thymidine incorporation), abundance and biomass of the epiphytic bacteria attached to detritus of Typha domingensis pens. in a tropical coastal lagoon. Hydrobiologia 357:17–26

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