

Top–down effects of a grazing, omnivorous minnow (*Campostoma anomalum*) on stream microbial communities

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Abstract: Top–down control exerted by macroconsumers can strongly affect lower trophic levels and ecosystem processes. Studies of effects on primary consumers in streams have been focused on algae, and effects on bacteria are largely unknown. We manipulated the density of an omnivorous, grazing minnow, the central stoneroller (*Campostoma anomalum*), in experimental stream mesocosms (treatments with 0, 1, 2, 3, 4, 5, 6, or 7 individuals) to understand consumer effects on algal and bacterial abundance (chlorophyll *a* [Chl *a*] extraction, bacterial cell counts, biomass measurements) and bacterial diversity and community composition (via Illumina MiSeq sequencing of the V4 region of the 16S ribosomal RNA gene). Increasing *C. anomalum* density reduced algal biomass until density reached ~2 fish (5 g fish biomass/m²), and higher fish densities did not affect algal biomass. Fish biomass did not affect bacterial cell counts. Biofilm organic matter decreased with increasing *C. anomalum* biomass. Bacterial community composition was not affected by fish biomass, but variation in community composition was correlated with shifts in bacterial abundances. Evenness of bacterial operational taxonomic units (OTUs) decreased with increasing *C. anomalum* biomass, indicating that bacterial communities exhibited a greater degree of OTU dominance when fish biomass was higher. These findings suggest that this grazing fish species reduces algal abundance and organic matter in low-nutrient streams until a threshold of moderate fish abundance is reached and that it reduces evenness of benthic bacterial communities but not bacterial biomass. Given the importance of biofilm bacteria for ecosystem processes and the ubiquity of grazing fishes in streams, future researchers should explore both top–down and bottom–up interactions in alternative environmental contexts and with other grazing fish species.

Key words: 16S rRNA sequencing, stream biofilms, *C. anomalum*, chlorophyll *a*, bacterial cell counts, AFDM, Konza Prairie

Consumer foraging strongly influences the structure of bottom trophic levels in terrestrial (Rooney and Waller 2003, Peschel et al. 2015), marine (Myers et al. 2007), and freshwater (Power 1992, Vanni 2002) ecosystems, modifying ecosystem stability and function (Pringle and Hamazaki 1997). High consumer densities can substantially reduce prey diversity and biomass (Jaschinski et al. 2010), which may have important consequences for nutrient cycling, primary and secondary production (Moriarty et al. 1985, Loreau et al. 2001), or decomposition rates (Peschel et al. 2015). Trophic interactions leading to foodweb changes in freshwater systems emphasize the effects of predatory (McQueen et al. 1989, Hoeinghaus and Pelicice 2010, Sullam et al. 2017), omnivorous (Lodge et al. 1994, Flecker et al.

2002, Taylor et al. 2012a), and herbivorous macroconsumers (Creed 1994) on foodweb compartments. Primary consumers have direct interactions with bottom trophic levels through grazing, which may reduce primary producer biomass (Taylor and Schiel 2010, Reynolds et al. 2014, Martin et al. 2016) and affect primary producer and bacterial diversity (Birtel and Matthews 2016). Conversely, grazers can stimulate microbial growth or productivity via nutrient remineralization through excretion (Vanni 2002, Hall et al. 2007, Taylor et al. 2012b, Berga et al. 2015, Capps and Flecker 2015), species replacement of grazing-sensitive algae with grazing-resistant forms (Steneck and Dethier 1994, Abe et al. 2006), or by removing benthic sediments that limit shade-intolerant N-fixing algae (Flecker 1996)

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and, thereby, altering the balance of autotrophy and heterotrophy (Taylor et al. 2006). However, grazing consumers may impart relatively weak effects on algal communities (Longmuir et al. 2007, Murdock et al. 2011) because of the diversity of dietary resources available or limitation of palatable algal species present locally, suggesting the strength of these trophic interactions may be context dependent (Garcia et al. 2015). However, because of the high palatability of many algae compared to plants, aquatic ecosystems typically have strong trophic interactions between grazers and primary producers (Shurin et al. 2002, Hillebrand 2009) making them sound model systems to study the dynamics of primary consumer–producer interactions.

Intermittent streams experience periodic floods and droughts resulting in nonequilibrium conditions (Dodds et al. 2004), which can select for resilient aquatic communities (Leigh and Detry 2017). The strength of top–down effects is tightly linked to recovery of consumers and their resources in high-disturbance ecosystems (Snyder 2009, Murdock et al. 2011). Larger animals, such as fish, are mobile, can seek refuge during flooding events, and therefore, populations recover to predisturbance densities within days or weeks (Franssen et al. 2006). Conversely, depending on disturbance magnitudes, microbial communities are scoured after flooding and may require months (Veach et al. 2016) to return to a predisturbance state. Thus, their successional development is immediately affected by multiple consumer-driven interactions, such as cell removal via consumption, bioturbation, or nutrient inputs from excretion (Gido et al. 2010, Taylor et al. 2010). These interactions can substantially change microbial successional trajectories, thereby altering ecosystem processes, such as primary production and N uptake rates during early succession (Murdock et al. 2011). Long-term precipitation predictions suggest greater annual precipitation and more frequent extreme precipitation events in the future for regions in North America (IPCC 2014). Thus, the incidence of high-pulse disturbances is expected to be more frequent, and stream biota will have a greater probability of being in an early successional state. Top–down effects may be important drivers of both microbial community dynamics and function under this scenario. Therefore, understanding consumer effects during early microbial succession is critical for predicting future stream ecosystem state changes.

Biofilms are diverse assemblages of microbes in an extracellular polysaccharide matrix (EPS) attached to a surface. In streams, which are highly heterogeneous over small spatial scales, biofilms form diverse microarchitectures that can create fine-scale variation in ecosystem processes (Battin et al. 2003). Bacteria and algae are in close association with each other and control energy flow and biogeochemical cycling in aquatic ecosystems. They com-

monly interact via facilitation—algal exudates supply C to bacteria (Naeem et al. 2000) and, thereby, can support more abundant and diverse bacterial populations (Cole 1982, Besemer et al. 2009). Furthermore, biotic (e.g., herbivory) or abiotic (e.g., light, limiting nutrients) conditions that affect algal recruitment, biomass, or alter relative abundances of algal functional guilds (e.g., filamentous green algae) may act as environmental filters for bacterial taxa (Dodds et al. 1996). Consumers forage directly on both algae and bacteria and may influence bacterial recruitment indirectly by removing suitable habitat (e.g., algal filaments), subsequently lowering bacterial biomass (Moriarty et al. 1985, Battin et al. 2003), shifting community structure, and reducing bacterial diversity. Conversely, when grazing removes algal biomass and strongly alters algal communities, bacteria may outcompete their algal neighbors for space and resources, thus preventing significant algal accrual and growth (Cole 1982, Fukami et al. 1991). These direct and indirect linkages between grazing fish and stream biofilms may drive microbial successional trajectories because of the co-occurrence of algal and bacterial species in stream ecosystems.

Stream ecologists have acknowledged the importance of bacteria in nutrient processing (Lawson et al. 1984, Findlay et al. 1986) and microbial control over resource quality for higher trophic levels (Kaushik and Hynes 1971), but the molecular tools needed to evaluate microbial taxonomic diversity have been available only recently. Understanding of top–down effects on microbial diversity is needed to bridge the gap in our understanding of consumer regulation of biofilm structure and function in changing environments. We manipulated the density of *Camptostoma anomalum*, and thereby grazing pressure on developing stream biofilms, to test the magnitude of top–down effects on 2 lower trophic levels: algae and bacteria. Central stone-rollers are omnivores that forage primarily on algae and detritus (Evans-White et al. 2003), are widely distributed throughout North America, and can directly and indirectly affect many structural and functional stream ecosystem properties (Matthews et al. 1987, Gido et al. 2010, Taylor et al. 2012a). However, an understanding of how these ubiquitous grazers (or other similar species) affect heterotrophic microbial communities (e.g., bacteria) is lacking, particularly in intermittent streams where stonerollers and other fish species concentrate during periods of natural drought (Franssen et al. 2006). We hypothesized that higher *C. anomalum* density would: 1) decrease algal biomass, bacterial biomass, and biofilm organic matter directly via grazing; and 2) decrease bacterial diversity subsequent to reductions in biofilm thickness and homogenization of biotic (algal community structure) and abiotic (e.g., chemical) gradients within the biofilm. Therefore, these effects will shift the taxonomic composition of bacterial communities at higher *C. anomalum* densities.

METHODS

Experimental design

In autumn 2013, we used 24 outdoor experimental mesocosms at Konza Prairie Biological Station (KPBS) to manipulate algal fish density and assess grazer density effects on freshwater biofilm communities. Each mesocosm consisted of a 2.54-m² circular pool (1136-L tank) connected to a 0.84-m² rectangular riffle (Matthews et al. 2006). Water was supplied continuously to all mesocosms from a low-nutrient groundwater spring (mean 35 µg/L NH₄-N, 10 µg/L soluble reactive P [SRP], KPBS–Long-Term Ecological Research, unpublished data; Martin et al. 2016) at ~1000 L/d and recirculated by electric trolling motors to mimic natural stream current with an average discharge of ~10 L/s (Bertrand and Gido 2006). All mesocosms were lined with gravel and filled with spring water for 14 d prior to the start of the experiment to allow benthic communities to develop. Microbial inocula are provided by residual communities from previous experiments, whereas macroinvertebrates colonize mesocosms from a nearby stream segment (<300 m) via egg deposition by aerial females.

On 2 October 2013, we placed 1 rectangular, hardware-cloth mesh enclosure (91 × 61 × 76 cm [length × width × height], 3-mm mesh size) in the pool of each mesocosm (Troia and Gido 2014). We used aquarium-grade silicone (Dow Corning Chemical Company, Auburn, Michigan) to attach autoclaved unglazed ceramic tiles (4.8 × 2.8 cm) to patio stones (40.6 × 40.6 cm) on the bottom of each enclosure. We covered open spaces on the enclosure bottoms with gravel. On 5 October 2013, we randomly assigned each mesocosm to a fish density treatment (0 individuals [no-fish control], 1, 2, 3, 4, 5, 6, or 7 individuals) to yield 3 replicate mesocosms for each density treatment. We collected *C. anomalum* from Carnahan Creek (~40 km from KPBS) with the aid of a DC-pulsed backpack electroshocker and randomly placed individuals 50 to 60 mm long (mean 55.6 mm) to reach desired densities. We placed fish in the pools of each mesocosm to minimize habitat heterogeneity for grazers by denying them access to riffles.

On 2 November 2013, 30 d after stonerollers were introduced to the mesocosms, we randomly sampled tiles from each enclosure for chlorophyll *a* (Chl *a*), bacterial cell count, and ash-free dry mass (AFDM). We placed tiles in separate Whirl-Pak[®] bags (Nasco International, Fort Atkinson, Wisconsin) and kept them on ice for transport to the laboratory. We also randomly sampled gravel from each enclosure for Chl *a* and AFDM to test whether microbial colonization and biomass differed between natural substrata and tiles. After sampling, we euthanized stonerollers from each mesocosm with a lethal dose of MS-222 (tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, WA) and preserved them in 10% formalin. We eviscerated and weighed fish to measure fish biomass in

each mesh enclosure, and calculated Fulton's condition factor, *K* (Fulton 1904), to test whether fish biomass was correlated with fish condition because density-dependent growth may occur.

Microbial abundance and estimation of AFDM

We subsampled 2 tiles and rocks from each mesocosm for Chl *a* and AFDM and 1 tile for bacterial cell counts. We froze tiles for Chl *a* analysis at -20°C upon arrival at the laboratory and analyzed them within 2 wk. We placed tiles in 95% ethanol : H₂O, heated at 78°C for 5 min and refrigerated 4°C for ~12 h (Sartory and Grobbelaar 1984). We used spectrophotometry to analyze the extract (Hitachi High Technologies America, Schaumburg, Illinois) according to standard methods (APHA 1995). We corrected Chl *a* values by tile surface area.

We preserved each tile for bacterial cell count in 3% formalin. We sonicated these tiles in an ultrasonic bath (Fisher Scientific FS-20, Hampton, New Hampshire) for ~10 min to remove biofilms, and we counted bacterial cells within 2 wk of collection. We incubated a 1-mL subsample of preserved biofilm with 4',6-diamidino-2-phenylindole (DAPI) nucleic acid stain (5 mg/mL) for 5 min in the dark and filtered the subsample through a polycarbonate membrane (Whatman Nuclepore, 0.2-µm pore size; GE Healthcare Companies, Pittsburgh, Pennsylvania). We counted cells in 10 to 15 optical fields under an epifluorescent microscope (Nikon Labophot-2; Nikon Corporation, Tokyo, Japan).

We estimated AFDM by removing biofilms from each tile via sonication, drying the slurry at 60°C for ~48 h, and combusting it in a muffle furnace at 450°C for 4 h. We calculated the difference in mass between dry and combusted biofilms. Lastly, we calculated the autotrophic index ($[\text{mg AFDM}/\text{cm}^2]/[\text{mg Chl } a/\text{cm}^2]$) to assess whether an increase in *C. anomalum* density shifts the ratio of autotrophic to heterotrophic biomass.

DNA extraction and Illumina MiSeq analysis

We used a Qiagen DNeasy Maxi Kit (Qiagen, Venlo, The Netherlands) to extract genomic DNA from 1 randomly sampled tile/mesocosm. We modified the manufacturer's protocol slightly by sonicating tiles in cell lysis solutions for ~10 min to remove biofilms. We used a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, Delaware) to quantify extracted DNA and standardized sample DNA to 2 ng/µL.

We used a 2-step polymerase chain reaction (PCR) approach to prevent a 3'-end amplification bias caused by the use of DNA tags (Berry et al. 2011). We used 515F and 806R primers to amplify the 16S ribosomal (r)RNA V4 region (Caporaso et al. 2012). We amplified each sample in triplicate independent 50-µL PCRs with a Mastercycler (Eppendorf, Hamburg, Germany) and included a negative

control to confirm that the PCRs were contamination free. PCR reactions consisted of 2 μ M of forward and reverse primers, 10 ng of template DNA, 25 μ L Phusion High-Fidelity Master Mix (New England Biolabs, Ipswich, Massachusetts), and 10 μ L of molecular-grade water. PCR conditions were 5 min denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 1 min, annealing for 30 s at 50°C, extension for 1 min at 72°C, with a final extension for 10 min. Secondary PCRs were amplified as above with 10 μ L of primary PCR solution as template DNA, except that we used only 5 cycles and included a reverse primer with a 12-basepair (bp) unique multiplex identifier tag (MID-806R; Table S1). Both primary and secondary PCRs were visualized on a 1.5% agarose gel (mass/volume). We pooled secondary PCRs per experimental unit and cleaned them with Agencourt AMPure (Beckman-Coulter, Pasadena, California) per manufacturer's directions except we used a 1 : 1 ratio of AMPure XP bead solution to PCR volume to discriminate against small DNA fragments and remove residual primers. PCR amplicons were sequenced with Illumina MiSeq (MiSeq Reagent V2 Kit, 2 \times 250 cycle; Illumina, San Diego, California) at the Integrated Genomics Facility at Kansas State University (Manhattan, Kansas).

Bioinformatic and statistical analysis

We used mothur (version 1.33.1; Schloss et al. 2009) to process sequences. Paired-end .fastq files were contiged and sequences with any ambiguous bases, >1 mismatch to primers, >1 mismatch to the MID and homopolymeric regions >8 were removed. We aligned remaining sequences against the SILVA reference database (Pruesse et al. 2007). To minimize sequencing-platform-generated errors, we clustered $\geq 99\%$ -similar sequences (pre.cluster) (Huse et al. 2010) and further screened remaining sequences for chimeras (UCHIME; Edgar 2010). We then aligned sequences to taxonomic affinities with the Naïve Bayesian Classifier (Wang et al. 2007) with a bootstrap threshold of 80% against the Ribosomal Database Project (RDP) training set (version 9). We removed sequences not assigned to Domain Bacteria, including Archaea, mitochondria, and chloroplasts, and calculated a pairwise distance matrix to cluster OTUs at a 97% sequence similarity with a nearest-neighbor joining method. We removed singletons (abundance = 1 across all samples) and rarified samples to 32,000 sequences/sample. The final data set had 768,000 sequences and 2104 OTUs. The .fastq files are available at the Sequence Read Archive (NCBI SRA BioProject: PRJNA350128 and Study Accession SRP113016).

We calculated Good's coverage (ratio of local OTU singletons to total number of sequences in a sample), OTU richness (S_{obs}), the complement of Simpson's Diversity ($1 - D: 1 - \sum p_i^2$), and Simpson's Evenness ($E: 1 - \sum p_i^2 / S_{\text{obs}}$),

with p_i representing the frequency of each OTU in a sample, iteratively in mothur (Schloss et al. 2009) after normalizing at 32,000 sequences/sample. We chose to standardize samples based on library size to reduce bias across samples that vary in sequence count (Gihring et al. 2012).

We chose to use fish biomass instead of fish density in statistical models because of variation in body size within density treatments (Fig. S1), which can affect grazing intensity. We used a linear regression model to test whether fish body condition (i.e., Fulton's K) was correlated with fish biomass (function `lm` in R; version 3.3.1; R Project for Statistical Computing, Vienna, Austria). We also used linear regression models to test whether microbial abundance (i.e., algal biomass, bacterial abundance), AFDM, and the AI varied with fish biomass (function `lm`). If a significant correlation was detected, we used a maximum likelihood estimation approach to test whether breakpoints existed in relationships between fish biomass (independent variable) and response variables (function `segmented` in the *segmented* package, Muggeo 2008). First, we estimated the breakpoint based on an iterative search to find the model with the lowest mean squared error (MSE; Crawley 2012). We then used the breakpoint that resulted in the lowest MSE as the breakpoint in segmented regression models (`psi` in `segmented` function, *segmented* package; Muggeo 2008). We report statistics for broken-line (*segmented*) regressions only if a breakpoint was detected. Furthermore, to test whether substratum differences in microbial colonization and growth existed, we used a nonparametric 2-sample Kolmogorov-Smirnov test (`ks.test` in *stats* package) for both Chl *a* and AFDM associated with tiles and gravel rocks. We also used linear regression models to test whether bacterial richness (OTU S_{obs}), diversity (complement of Simpson's Diversity), and evenness (Simpson's Evenness) and the abundance of bacterial taxa (i.e., phyla, genera, and OTU frequency) were correlated with fish density and microbial abundance (function `lm`). We did not include AFDM as an explanatory variable in these models because it was strongly correlated with Chl *a* (Pearson's $r = 0.87$). We excluded phyla, genera, or OTUs that did not occur in ≥ 2 samples from these analyses. We implemented a false discovery rate (FDR) correction for bacterial phyla, genera, and OTU regression models.

We identified differences in bacterial community composition across treatments with differing fish densities by calculating Bray-Curtis distances and visualized differences with non-metric multidimensional scaling (NMDS, function `metaMDS` in the *vegan* package; Oksanen et al. 2015). We initially set the number of NMDS dimensions to 2 and the maximum number of random starts to 20. These configurations produced a stress value of 0.19, and observed dissimilarity was strongly related to ordination distance as assessed by a Shepard's plot (function `stressplot` in *vegan*). Thus, we considered the ordination with 2 dimensions adequate. We

used a permutational multivariate analysis of variance (PERMANOVA; function *adonis* in *vegan* package; Anderson 2001, Oksanen et al. 2015) to partition variation in bacterial community composition among fish biomass, microbial abundance, and AFDM. We also used a multiple linear regression model (function *lm*) with NMDS axis scores (2 dimensions, therefore 2 multiple regression models) as the dependent variable and fish biomass, microbial abundance, and AFDM as the independent variables to further confirm which of these significantly correlated with bacterial community composition. The threshold for Type I error rate is 0.05.

RESULTS

Microbial abundance and bacterial diversity

Fish body condition (Fulton's K) was not correlated with total fish biomass across treatments (adjusted [adj.] $R^2 = 0.08$, $p = 0.07$; Fig. S2). In general, algae and bacteria differed in their responses across the gradient of grazing fish biomass. A breakpoint was detected for Chl *a* as a function of fish biomass (breakpoint = 5.83 g/m^2 , 95% CI [2.09, 9.58]). Chl *a* was negatively correlated with fish biomass until 5.83 g/m^2 (or ~ 2 *C. anomalum* individuals at 50–60 mm length), but exhibited no significant correlation with grazer biomass when biomass was $>5.83 \text{ g/m}^2$ (Table 1, Fig. 1A) indicating that after a threshold of ~ 2 fish, algal biomass did not change with increasing fish biomass. AFDM was negatively correlated with fish biomass ($p < 0.01$; Table 1, Fig. 1B). However, in contrast to the nonlinear relationship between algal biomass and fish biomass, no breakpoint was detected for AFDM. Treatments with no fish had $2.3\times$ greater algal biomass (mean \pm SD = $1.44 \pm 0.78 \text{ } \mu\text{g/cm}^2$) and $1.5\times$ greater biofilm AFDM ($0.36 \pm 0.15 \text{ g/cm}^2$) compared to the highest *C. anomalum* den-

sity treatments (7 fish: $0.63 \pm 0.23 \text{ } \mu\text{g/cm}^2$ Chl *a*; $0.11 \pm 0.04 \text{ mg AFDM/cm}^2$). Contrary to our hypothesis, bacterial abundance was low and variable across grazer density treatments ($p = 0.29$; Table 1, Fig. 1B), ranging nearly an order of magnitude (2.8×10^3 to 1.8×10^4 cells/cm²), and was not correlated with fish biomass (Fig. 1C). Last, the AI was also variable (range: 100–500) and was not correlated with fish biomass ($p = 0.08$; Table 1, Fig. 1D), indicating the ratio of autotrophic to heterotrophic biomass within biofilms did not significantly change with increasing grazing fish biomass.

We also compared natural substrata and tiles used for biofilm colonization in our study. Unlike tile Chl *a*, rock Chl *a* was not correlated with grazer biomass ($p = 0.45$). However, like tile AFDM, rock AFDM was negatively correlated with grazer biomass ($F = 10.05$, Adj. $R^2 = 0.41$, $p < 0.01$). Rock Chl *a* ($D = 0.83$, $p < 0.01$) was $3.8\times$ greater than tile Chl *a*, whereas rock AFDM did not differ from tile AFDM ($D = 0.36$, $p = 0.14$) suggesting that algal communities did not colonize tiles as readily as natural stream substrata. Tile biofilms had low Chl *a*, but they had concentrations similar to those on these tiles in natural streams in this region (Veitch et al. 2016).

Good's coverage was high overall (0.992 ± 0.001), indicating bacterial communities were adequately sampled. Bacterial OTU richness ranged between 597 and 796 OTUs across experimental units and was not influenced by any of the explanatory variables (full model: $p = 0.71$; Table 2, Fig. 2A). Bacterial diversity was not correlated with explanatory variables (full model: $p = 0.09$; Table 2, Fig. 2B). The full multiple regression model included microbial abundance and fish biomass as explanatory variables and was weakly significant ($p = 0.08$), but bacterial evenness was negatively correlated with fish biomass ($p = 0.04$; Table 2, Fig. 2C).

Table 1. Linear regression statistics for biofilm microbial abundance, organic matter, and autotrophic index (AI) with fish biomass. Estimates represent the intercept value for all "Intercept" coefficients and the regression slope for "Fish biomass" coefficients. NA is given for breakpoint regression full model statistics because these are not computed for the segmented maximum likelihood estimation procedure implemented. BP = breakpoint, AFDM = ash-free dry mass.

Response variable	Coefficients	Estimate	<i>T</i>	<i>p</i>	Full model statistics		
					<i>F</i>	Adjusted R^2	<i>p</i>
Chlorophyll <i>a</i>	Intercept	1.28	834	<0.01	NA	0.37	NA
	Fish biomass < BP	-0.12	-2.49	0.02			
	Fish biomass > BP	0.01	0.61	0.27			
Bacterial abundance	Intercept	9631.8	7.27	<0.01	1.18	0.01	0.29
	Fish biomass	-127.0	-1.09	0.29			
AFDM	Intercept	0.29	7.04	<0.01	8.94	0.26	<0.01
	Fish biomass	-0.01	-2.99	<0.01			
AI	Intercept	300.49	9.50	< 0.01	3.35	0.09	0.08
	Fish biomass	-5.19	-1.83	0.08			

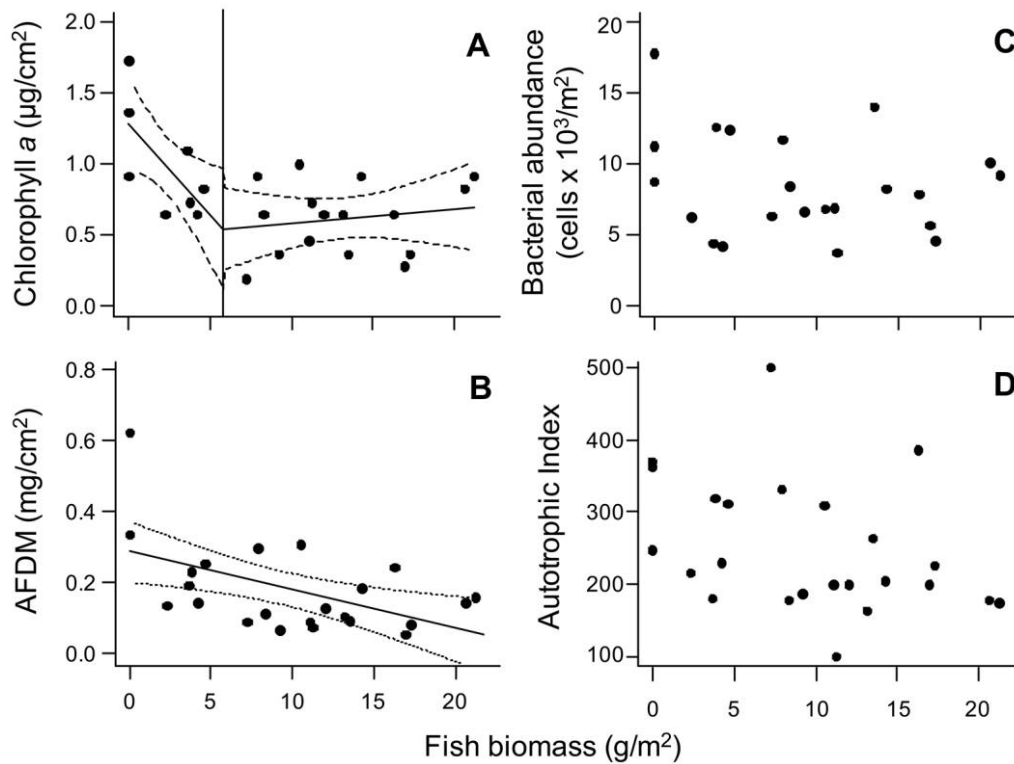


Figure 1. Chlorophyll *a* (A), ash-free dry mass (AFDM) (B), bacterial abundance (C), and autotrophic index (D) on tiles across fish biomass treatments. Linear regression lines and 95% confidence intervals (dashed lines) are shown for significant relationships. In panel A, the vertical line denotes the breakpoint.

Bacterial community composition and dominant taxa

Bacteroidetes (33.2% sequences), Betaproteobacteria (22.4%), Alphaproteobacteria (10.3%), Verrucomicrobia (6.4%), Gammaproteobacteria (4.2%), Planctomycetes (3.1%), Deltaproteobacteria (1.8%), Armatimonadetes (1.6%), and Acido-

bacteria (1.2%) were the most dominant (>1.0% relative abundance across samples) phyla or subphyla among all biofilm bacterial communities. Based on multiple regression analyses, most bacterial phyla and Proteobacterial subphyla did not vary with fish biomass, algal biomass, or bacterial

Table 2. Linear regression statistics for bacterial richness, diversity, and evenness correlated with fish biomass and microbial abundance. Estimates represent the intercept value for all “Intercept” coefficients and the regression slope for “Fish biomass” and microbial abundance coefficients.

Response variable	Coefficients	Estimate	<i>T</i>	<i>p</i>	Full model statistics		
					<i>F</i>	Adjusted <i>R</i> ²	<i>p</i>
Bacterial richness	Intercept	668.22	13.41	<0.01	0.46	-0.08	0.71
	Fish biomass	-0.04	-0.02	0.99			
	Chlorophyll <i>a</i>	-13.73	-0.31	0.76			
	Bacterial abundance	0.005	1.15	0.27			
Bacterial diversity	Intercept	0.93	76.41	<0.01	2.48	0.17	0.09
	Fish biomass	-0.002	-1.98	0.06			
	Chlorophyll <i>a</i>	-0.02	-1.52	0.15			
	Bacterial abundance	<0.001	1.8	0.09			
Bacterial evenness	Intercept	0.02	9.99	<0.01	2.71	0.20	0.08
	Fish biomass	<0.001	-2.17	0.04			
	Chlorophyll <i>a</i>	-0.002	-1.33	0.20			
	Bacterial abundance	<0.001	1.72	0.10			

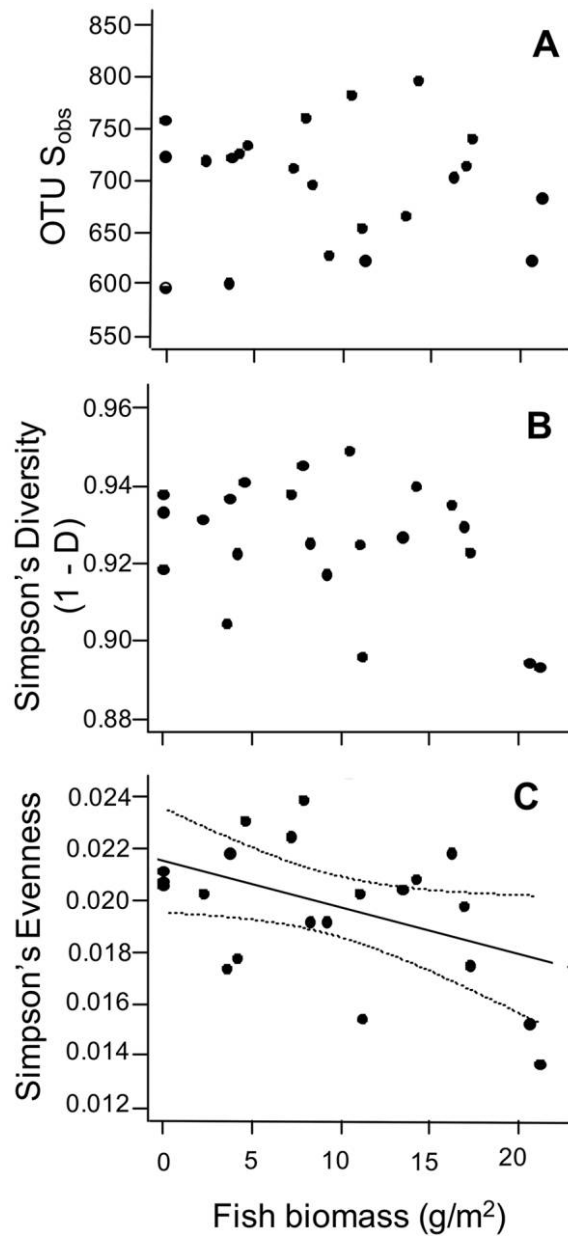


Figure 2. Bacterial operational taxonomic unit (OTU) richness (S_{obs}) (A), the complement of Simpson's diversity (D) based on OTUs (B), and Simpson's evenness based on OTUs (C) in biofilms across fish biomass treatments. Linear regression lines and 95% confidence intervals (dashed lines) are shown for significant relationships.

abundance except Armatimonadetes (candidate division OP10) and Parcubacteria (candidate division OD1). These taxa were positively correlated with bacterial abundance (Armatimonadetes full model: $F_{3,18} = 12.63$, Adj. $R^2 = 0.62$, $p < 0.01$; Parcubacteria full model: $F_{3,18} = 6.66$, Adj. $R^2 = 0.45$, $p = 0.04$). Chl a and AFDM were not correlated with the relative abundance of any bacterial phylum. No OTUs or genera differed across explanatory variables ($p > 0.05$). Based on the PERMANOVA analysis, bacterial com-

munity composition was not correlated strongly with fish biomass ($p = 0.08$), Chl a ($p = 0.10$), or AFDM ($p = 0.09$), but was correlated strongly with biofilm bacterial abundance ($p < 0.01$; Table 3, Figs 3, 4A–D). A large percentage of variation was unrelated to any measured response variables (residuals $R^2 = 0.70$; Table 3). This result was further confirmed by multiple regression analyses. Bacterial abundance ($T = 2.76$, $p = 0.01$) was the only variable correlated with NMDS axis 1 scores (full model: $F_{4,17} = 5.98$, Adj. $R^2 = 0.49$, $p < 0.01$, Fig. 4C). Axis 2 scores were not correlated with any explanatory variable (full model: $p = 0.37$).

DISCUSSION

Macroconsumers can control bottom trophic levels in stream ecosystems. For example, grazing fish can reduce algal filament lengths and change the composition and function of algal communities (Creed 1994, Flecker et al. 2002, Gido et al. 2010, Taylor et al. 2012a, Birtel and Matthews 2016). Yet, few investigators have focused on consumer effects on other lower trophic levels, such as bacterial communities (but see Taylor et al. 2012a for effects of *C. anomalum* on bacterial production). We manipulated the biomass of a grazing, omnivorous minnow to quantify the magnitude of top-down effects in biofilms during early succession and focused on the bacterial component using next-generation sequencing. We hypothesized that grazing consumers cause turnover in algal communities from grazing-sensitive microbial groups to less palatable groups and alter abiotic environmental gradients by removing biofilm mass, subsequently changing bacterial communities and lowering their diversity. Our results corroborate previous observations (Flecker et al. 2002, Hillebrand 2009, Gido et al. 2010) that grazing fish can reduce algal biomass and organic matter in stream biofilms. Support for our hypothesis that grazing fish would drive bacterial community shifts was inconclusive. Bacterial community evenness declined with increasing fish biomass, but no relationships with fish biomass were apparent for OTU richness (unique

Table 3. Results of the permutational multivariate analysis of variance of the effects of fish biomass, algal biomass, bacterial abundance, or biofilm organic matter (ash-free dry mass = AFDM) on biofilm bacterial community composition. The Bray–Curtis distance matrix was calculated for biofilm bacterial communities, and 999 permutations were used to calculate significance values.

Model factors	Pseudo- F	R^2	p
Fish biomass	1.51	0.06	0.08
Chlorophyll a	1.47	0.06	0.10
Bacterial abundance	2.84	0.12	0.001
AFDM	1.50	0.06	0.09
Residuals		0.70	

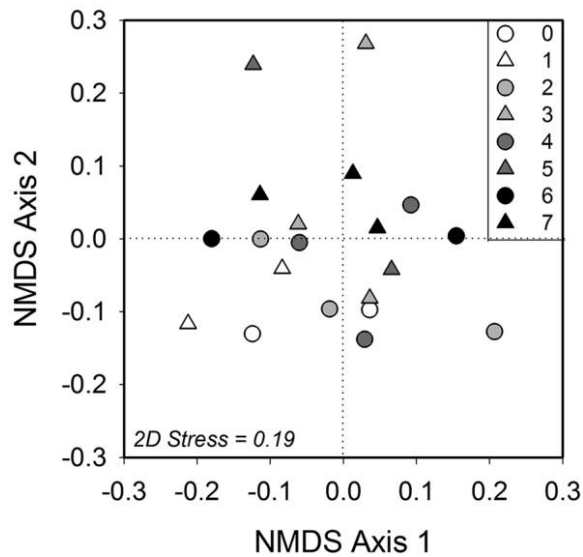


Figure 3. Nonmetric multidimensional scaling (NMDS) ordination of biofilm bacterial communities across fish biomass treatments. Treatments are denoted by a gradient of white to black (no fish treatments = white; highest fish density of 7 individuals = black) and symbols. No explanatory variables significantly influenced bacterial community composition except bacterial abundance. 2D = 2 dimensional.

number of OTUs within a community) or the relative abundances of dominant bacterial phyla, genera, or OTUs. Bacterial community composition was not correlated with fish biomass, but was correlated with bacterial abundance, a factor unaffected by increasing fish biomass. In sum, these observations suggest limited top-down effects by *C. anomalum* on streambed bacterial communities in low-nutrient streams.

Grazer effects on microbial biomass

We observed a threshold response of algal biomass to grazer density: the presence of grazers at low densities reduced algal biomass relative to the absence of grazers, but high grazer densities did not further reduce algal biomass. Two mechanisms could explain such nonlinear responses. First, interference competition among grazing fish could restrict the cumulative grazing effect of additional fish in high-density treatments. We did not detect a significant relationship between the density of grazing fish and body condition (Fig. S2), but body condition did generally decline with increasing density, potentially indicating density-dependent competition via intraspecific interference (Holbrook and Schmitt 1992, Grant and Imre 2005). Additional experiments, including diet analysis of *C. anomalum* in conjunction with resource pool estimation (microbial biomass, detrital pools, macroinvertebrates), are necessary to

discern the importance of this mechanism. Second, switching to alternative food resources when algal biomass is low may explain the observed threshold effects. This mechanism is more plausible than the first, given the minimal decline in body condition at high grazer densities. *Camptostoma anomalum* forages primarily on algae (Hargrave 2006), but these fish are omnivorous and also forage on macroinvertebrates and larval fish (Evans-White et al. 2003). Palatable macroinvertebrate taxa that frequently colonize stream mesocosms (Martin et al. 2016, Pennock and Gido 2016) were abundant throughout the current experiment (AMV, personal observation), and probably provided an alternative food source once algae were reduced below the threshold biomass. Grazers also may have switched to alternative algal resources. Algal biomass was, on average, 3× higher on the amorphous gravel that lined the experimental mesocosms than on the flat tiles. Grazers could have switched from algal resources on the tiles to those more abundant algal resources on the gravel once tile algae were reduced below the threshold biomass. A switch from sample tiles to amorphous gravel represents an artifact of these particular experimental mesocosms, but spatial heterogeneity in substratum size and shape is common on stream beds (Allan and Castillo 2007) and probably affects grazer–algae relationships.

Although grazing fish reduced algal biomass, we did not observe similar effects on bacterial abundance. The lack of effects on bacteria could be explained by 2 alternative, but not mutually exclusive mechanisms. First, a major component of epilithic biofilm biomass is bacterially produced EPS, which can be assimilated in amounts comparable to algae by small-bodied, grazing animals (Morales and Ward 2000). Removal of EPS by grazers does not necessarily translate to equal removal of bacterial biomass from substrata. Grazing snails, insects, and ostracods consume primarily EPS with no discernable reduction in bacterial biomass, potentially because these grazers do not consume primarily bacterial cells (Lawrence et al. 2002). Reductions in AFDM were observed, suggesting removal of detritus-based resources, but specific contributions to this resource pool (e.g., algae, bacteria, protozoa, fungi) are uncertain. This effect has been observed in other studies (Taylor et al. 2012a), suggesting that *C. anomalum* may select for tightly adhering algal communities at high densities with removal of detrital biomass, albeit not necessarily bacterial based. Second, bacterial recruitment and growth may occur rapidly after grazing removes algae from biofilms, resulting in no detectable net loss or gain in bacterial biomass (Lock et al. 1984). As such, the grazing fish in our study could possibly have consumed algae, EPS, and bacteria in similar amounts, but bacteria responded quickly to available resources (e.g., space, nutrients from overlying water column) in the absence of algal populations. This explanation is in agreement with the finding that consumers reduced both algal biomass and organic matter.

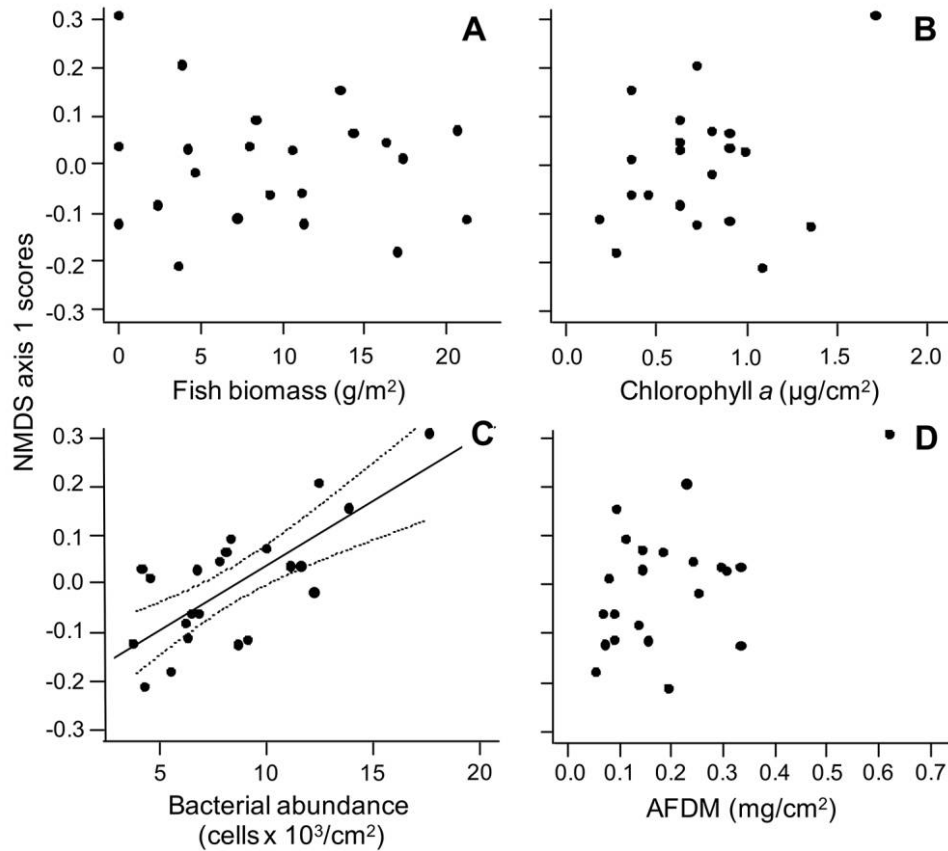


Figure 4. Nonmetric multidimensional scaling (NMDS) axis 1 scores across fish biomass (A), biofilm chlorophyll *a* (B), bacterial abundance (C), and ash-free dry mass (AFDM) (D). Linear regression lines and 95% confidence intervals (dashed lines) are shown for significant relationships.

Grazer effects on bacterial community composition

We tested whether grazers would reduce bacterial diversity and shift community composition while they decreased microbial biomass and biofilm organic matter. We hypothesized that grazers would reduce biofilm thickness as a result of algal removal and reduced environmental heterogeneity, thereby removing niche space for bacterial species to occupy. We observed mixed evidence for this hypothesis. Grazing intensity directly reduced bacterial OTU evenness, but did not affect OTU community composition. Community evenness often responds before richness to an environmental disturbance (e.g., grazing) as the relative abundances of sensitive species decline. This process eventually leads to local species extinction and a reduction in taxon richness over time (Chapin et al. 2000, Hillebrand et al. 2008). The negative correlation between evenness and grazer density may be merely the first indication of grazer-induced turnover in the bacterial community potentially because of reductions in biofilm environmental heterogeneity. Bacterial cell abundance did not decline with grazer density, so it is more likely that consumption of algal cells and detritus (i.e., AFDM) affected biofilm heterogeneity,

which led to changes in bacterial OTU dominance. Had we included treatments with higher grazer densities, continued the experiment longer, or done this experiment during summer when water temperatures and organismal metabolic activity are greater, a concomitant change in OTU richness may have resulted. Alternatively, an effect on Simpson's evenness does not necessarily translate into similar outcomes for diversity because these values are calculated independently (Smith and Wilson 1996, Magurran 2013) and may merely reflect changes in bacterial OTU dominance as biofilm microarchitectures change in response to grazing pressure.

In terms of community composition, bacterial communities primarily responded through changes in bacterial cell abundance, as evidenced by the shifting relative abundances of specific phyla and by the shifting of community composition with greater bacterial cell numbers, regardless of fish densities. Contrary to our hypotheses, processes regulating bacterial biomass recruitment and community assembly may be independent of other trophic levels (i.e., primary producers or consumers; Longmuir et al. 2007), which may lead to limited congruence between these groups

because of spatial scale dependencies (Westgate et al. 2014). For example, Longmuir et al. (2007) showed that no feedbacks existed between zooplankton, phytoplankton, and bacteria in lake pelagic habitats. Feedbacks do occur for *C. anomalum* and periphyton (Gido et al. 2010, Taylor et al. 2012a), but the interaction between bacterial taxa and this consumer is weak. Under other environmental conditions (eutrophic states, higher temperatures), algal–bacterial interactions in biofilms exposed to grazing pressure are important drivers of the balance between autotrophy and heterotrophy (Flecker 1996, Taylor et al. 2006). However, in oligotrophic streams during periods of low temperature, these interactions are limited.

Conclusions

Grazing fishes impart complex and context-dependent effects on the structural and functional characteristics of stream biofilms. We built on this knowledge in several key ways. First, by evaluating biofilm characteristics along a gradient of grazing densities, we identified a nonlinear response that reveals a complex relationship between intraspecific grazer interactions, resource availability, and physical characteristics of substrata. These findings provide a starting point for future experiments aimed at evaluating the implications for these context dependencies. Second, whereas previous studies have characterized effects of grazing minnows on biofilms during the warm season, we characterized this relationship in autumn. Our findings confirm that grazing minnows can impart strong top–down effects on algal biomass even when low temperatures limit energetic requirements of fish and growth rates of algae. Third, we used next-generation sequencing to dissect bacterial communities deeply to characterize grazer effects on the taxonomic composition of bacteria in stream biofilms. This component of the experiment revealed mixed results, but it provides preliminary information for additional studies aimed at understanding how top–down processes affect taxonomic composition of biofilms and the consequent implications for biogeochemical processes in stream ecosystems.

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