The Effects of Eutrophication on the Structure and Function of Microbial Biofilms

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Abstract:

Biofilms are the dominate form of microbial life in aquatic ecosystems and are responsible for performing a wide variety of ecosystem services including nutrient and organic matter processing and retention. Understanding how eutrophication impacts these communities is essential for ecosystem managers as many aquatic ecosystems are being enriched by anthropogenic activities. This study investigated the effects of eutrophication on biofilm productivity, community structure and diversity, and function. Increasing background dissolved organic carbon and nutrient concentrations by a factor of twelve increased biofilm ash free dry mass and the abundance of live cells ~ 22 and 200 fold respectively. Extracellular enzyme activities for five enzymes from the same samples showed a range of increase from ~ 300 times for phosphatase to 8000 times for N-acetylglucosaminidase. Enrichment decreased the bacterial diversity of these biofilms and resulted in a gradual shift in community structure that intensified from low to high enrichment. These data indicate that the productivity, community structure, and function of stream biofilm communities are highly responsive to eutrophication inputs.

Introduction:

Microbes are the most abundant organisms on Earth and play a central role in all global processes. While often overlooked due to their inconspicuous nature, microbes contain as much carbon and ten times more nitrogen and phosphorus than plants (Whitman et al. 1998), possess unique metabolic pathways essential to biogeochemical cycles (Schlesinger 1997), and represent a potentially massive pool of genetic diversity that is only now being explored (Curtis et al. 2006, Achtman and Wagner 2008). Recent methodological advances have given ecologists the tools to investigate the ecology of these vitally important organisms, revealing that microbes follow some of the general ecological patterns identified in plant and animal communities including latitudinal diversity gradients (Fuhrman et al. 2008), diversity-productivity relationships (Horner-Devine et al. 2003, Smith 2007), and taxa-area relationships (Green and Bohannan 2006, Prosser et al. 2007). While these studies have provided important insights into the ecology of microbes, very few have investigated the ecology of microbial biofilms, which are complex communities of microbes attached to solid surfaces. This constitutes a significant knowledge gap in microbial ecology because biofilms represent the dominate microbial community in all aquatic environments and are responsible for providing a wide variety of ecosystem services (Costerton et al. 1995, Watnick and Kolter 2000, Battin et al. 2003, Battin et al. 2007).

Over the past decade a significant body of biofilm research has revealed that microbial biofilm associations have many unique attributes not found in planktonic communities that may significantly affect the ecological patterns they display. The unique biofilm life stage begins when planktonic microbial cells adhere to solid surfaces. Attachment triggers a cascade of changes that ultimately result in the formation of an extremely complex and dynamic microbial community. This cascade begins as microbial gene expression patterns undergo a series of changes resulting in the aggregation of individual microbes into micro-colonies with unique physical structures (Watnick and Kolter 2000, Beloin and Ghigo 2005). These structures are determined by the interaction of the surrounding environmental conditions, selection by these conditions for some members of the microbial community, and gene regulation of the selected biofilm members (Hall-Stoodley et al. 2004, Kolter 2005). As biofilms mature, they form into a dense layer of microbes and extracellular polymeric substances (EPS). This layer protects biofilm organisms from predators (Webb et al. 2003) and provides a stable micro-environment, insulating the inhabitants from external physical conditions such as pH and temperature fluctuations, ultraviolet light, desiccation, and toxic or antimicrobial substances (Webb et al. 2003, Hall-Stoodley et al. 2004). Physical and chemical gradients form as biofilms grow and thicken, producing niches for a wide variety of metabolic lifestyles (Costerton et al. 1995). The occurrence of multiple niches in close proximity to one another facilitates efficient physiological and metabolic cooperation between bacterial species, with waste products from one metabolic pathway used as energy sources by other microbes (Costerton et al. 1995, Davey and O'Toole 2000). In addition to sharing metabolic by-products, microbes living in biofilms participate in several forms of intercellular communication including quorum sensing (Hense et al. 2007), intercellular signaling that can produce lethal interactions between species (Watnick and Kolter 2000), programmed cell death among members of the same species (Webb et al. 2003), and lateral gene transfer within and among species (Watnick and Kolter 2000, Parsek and Fuqua 2004). Biofilm microbes are also capable of actively releasing planktonic cells into the surrounding aquatic environment, allowing microbes to colonize new or more favorable niches and environments (Watnick and Kolter 2000, Webb et al. 2003, Hall-Stoodley et al. 2004, Parsek and Fuqua 2004). These complex physical, chemical, and social conditions that occur in biofilms have led some researchers to describe biofilm communities as multicultural, highly differentiated and complex communities analogous to modern cities (Watnick and Kolter 2000), while other groups have compared biofilms to multicellular organisms (Costerton et al. 1995). Clearly, biofilms have emergent properties that make this form of microbial association unique when compared to other microbial communities (Parsek and Fuqua 2004).

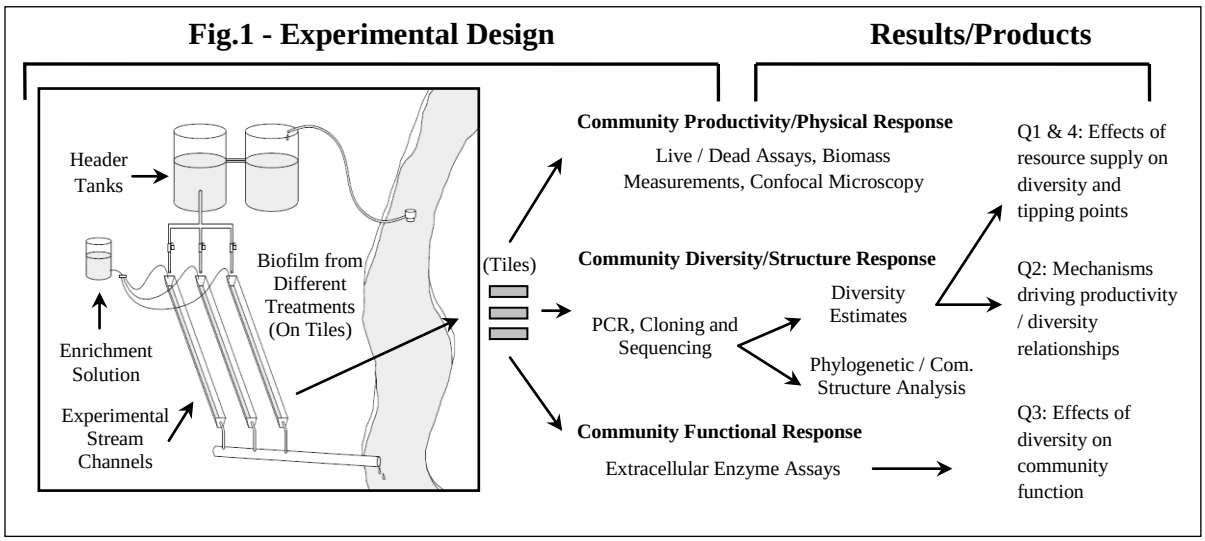
Understanding how the unique characteristics of biofilms discussed above impact the ecology of these complex communities is an essential and understudied area of microbial ecology. A specific knowledge gap that was identified in a recent review of microbial ecological theory is how the productivity of microbial communities affects two important ecological characteristics of these associations: community diversity/structure, and community function (Prosser et al. 2007). From a theoretical perspective, productivity/diversity (Waide et al. 1999, Mittelbach et al. 2001) and diversity/function (Loreau et al. 2001, Hooper et al. 2005) relationships have been investigated in hundreds of plant and animal studies, providing an excellent opportunity to compare the ecology of microbes to multicellular organisms. The relationship between microbial productivity and diversity and function is practically important because biofilm productivity is known to be highly responsive to nutrient availability (Costerton et al. 1995), and the freshwater aquatic ecosystems where biofilms are the dominant form of microbial life (Geesey et al. 1978) have been disproportionately affected by anthropogenic eutrophication (Alexander et al. 2000, Peterson et al. 2001). Little is known about how these inputs affect biofilm structure, and function. Negative eutrophication impacts on microbial biofilms could have serious implications for the important ecosystem services these communities provide in aquatic environments (Battin et al. 2003).

This purpose of this project was to answer four specific research questions: **Q1:** How does resource supply/eutrophication affect stream biofilm community productivity, **Q2:** Do changes in productivity affect biofilm community diversity and structure, **Q3:** Do changes in community diversity or structure affect biofilm function, and **Q4:** Do enrichment thresholds exist that if crossed, result in rapid changes in community structure and function?

Methods:

General Experimental Design:

Heterotrophic microbial biofilms were grown in the dark in fifteen experimental stream channel mesocosms. These mesocosms have been shown to be an effective model system for studying natural microbial biofilm dynamics (Singer et al. 2006). The channels were lined with removable unglazed ceramic tiles, supplied with river water to ensure colonization by natural bacterial populations, and continuously enriched with a stochiometrically balanced solution of dissolved organic carbon (DOC), nitrate and phosphate to create a productivity gradient (Fig. 1). The enrichment levels were based on the background concentration of DOC in the stream water and included no enrichment in control channels, two (2X), four (4X), eight (8X) and twelve times (12X) the background DOC concentration (1.5 mg l^{-1} DOC). Three replicate channels were used for each level of enrichment. After three weeks of growth, the time necessary to produce a mature biofilm, samples were collected to assess the impacts of the enrichment (Fig. 1).



Measuring the Effects of Eutrophication on Biofilm Productivity and Physical Structure:

The affects of eutrophication on biofilm productivity were assessed by quantifying the biomass (measured as ash free dry mass), and the relative abundance of live and dead cells from biofilms from the different enrichment treatments. Biomass was measured by removing three replicate tiles with biofilm growth from each channel, scraping the tiles, and transferring the biomass to ashed, tared glass fiber filters and aluminum tins. Filters were dried at 80°C, reweighted to calculate dry weights, and ashed at 500°C for three hours. Ashed filters were reweighted and ash free dry mass (AFDM) was calculated as the difference between dry mass and ash mass. The relative abundance of live and dead cells was also assessed for triplicate samples from each channel. A BacLight Kit (Invitrogen, Eugene, OR) containing propidium iodide and SYTO® 9 stains which stain dead and live cells respectively, was used to stain the biofilm samples. The stains were first checked for linearity of fluorescence over the biofilm cell densities to be analyzed. Samples were then homogenized in a bicarbonate buffer solution, 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 was added to the microplate wells. Samples were incubate at room temperature in the dark for ~15 minutes and then read on a fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA) 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 from the AFDM and live/dead measurements were normalized by the area of the tile sampled and were corrected for dilution where appropriate. The effect of eutrophication on the physical structure of biofilms was assessed using confocal microscopy. Briefly, a single biofilm sample from each channel was placed in a plastic tray while still submerged in the channel, placed on ice, and transported 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 5x objective on a LSM 510 confocal microscope (Carl Zeiss).

Measuring the Effects of Eutrophication on Biofilm Function:

The affects of eutrophication on biofilm function were assessed by measuring the activity of five hydrolytic extracellular enzymes: α-glucosidase (ALPHA), β-glucosidase (BETA), N-acetylglucosaminidase (NAG), phosphatase (PHOS), and leucine aminopeptidase (LEU). Potential activities were measured using coumarin and methylumbelliferon linked substrates that fluoresce when the substrate is cleaved by extracellular enzymes. Triplicate samples were run from each channel, and each sample was replicated 16 times using 200 µl sample aliquots with 50 µl of 200 µM substrate. Each plate also contained reference standards, substrate controls, and sample controls. Samples were incubated at room temperature in black, 96-well microplates. Fluoresce was measured periodically for up to 19 hours using a fmax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA) 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 nM substrated converted per hour per cm^2 of tile.

Measuring the Effects of Eutrophication on Biofilm Community Structure and Diversity:

Bacterial 16S rRNA gene sequences were amplified using the bacteria-specific forward primer 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1492R 5'-GTTTACCTTGTTACGACTT-3' in 50 ul reactions containing 5 µL 10X buffer (Promega Buffer B w/ 1.5 mM $MgCl_2$), 12.5 mM each dNTP (BioLine USA, Inc.), 20 pmol each of 8F and 1492R primers, 2.5 U Taq polymerase (Promega U.S.), and approximately 50 ng of DNA. The PCR thermal cycling (ABI GeneAmp 2700, Applied Biosystems, Foster City, CA) consisted of 30 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C. The amplified 16S rRNA genes were gel purified using a DNA Purification Kit

(MoBio Laboratories, Carlsbad, CA) and cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). One hundred and ninety two clones per library were sequenced using either M13 or 8F primers.

Biofilm community 16S rRNA gene sequence data was checked for quality using CodonCode Aligner. Sequences greater than 500 bp with a quality score above 500 were exported to Greengenes (http://greengenes.lbl.gov) for alignment (NAST Alignment Tool (8)). A distance matrix of the aligned sequences was generated in ARB (26). This matrix was analyzed in DOTUR (33) (http://www.plantpath.wisc.edu/fac/joh/dotur.html) to divide sequences into operational taxonomic units (OTUs) within each snail species using a 97% DNA sequence similarity cutoff, and to generate rarefaction curves for each sample.

The phylogeny of the bacterial 16S rRNA genes from the eight snail samples was analyzed using UniFrac (23, 24). Briefly, all aligned, high quality sequences were added using the parsimony add in ARB (26) to a backbone phylogenetic tree of 6634 bacterial 16S rRNA gene sequences (20). This tree was imported into UniFrac to calculate the UniFrac metric which is defined as the phylogenetic distance between sets of taxa in a tree calculated as the percentage of branch length that leads to descendants from only one of a pair of environments represented in a single phylogenetic tree (UniFrac Metric) (24). This UniFrac metric was then used to perform a variety of tests. Environment Distance Matrices (EDM) were calculated to measure distances between all sample pairs in a tree(23). These EDMs were then used to hierarchically cluster samples using an Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm (23). Jackknifing was used to assess confidence in the nodes of the UPGMA tree (23). The Environment Distance Matrices were also used to perform a principal coordinate analysis (PCoA) (23).

Results:

Effects of Eutrophication on Biofilm Productivity and Physical Structure:

The eutrophication gradient created in this experiment resulted in dramatically increased productivity in the high (8X and 12X) enrichments (Fig. 2). Mean AFDM values in the control, 2X and 4X enrichments ranged from ~ 0.1 to 0.25 mg cm^{-2} while values in the 8x and 12X enrichment ranged from ~1.0 to 1.25 mg cm^{-2} (Fig. 2). The eutrophication gradient also increased the relative areal abundance of live and dead biofilm cells with a 10, 53, 213, and 193 percent increase in live cells and a 10, 34, 126, and 132 percent increase in dead cells across the 2X, 4X, 8X, and 12X eutrophication enrichments respectively (Fig. 2).

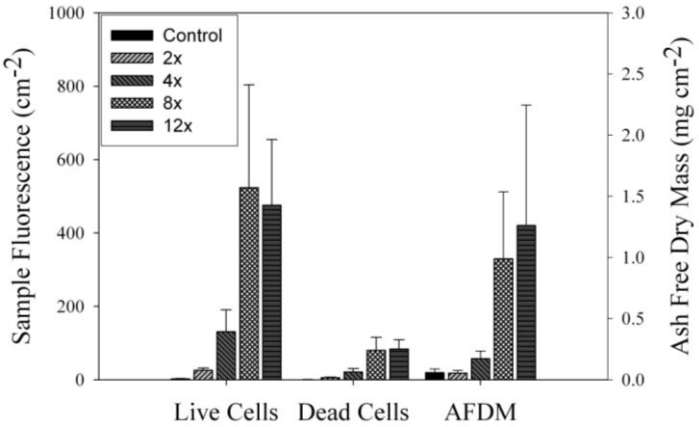


Figure 2: Relative live/dead cell abundance (fluorescence cm^{-2}) and ash free dry mass (mg cm^{-2}) for the five treatments (ordered Control through 12x).

The eutrophication gradient also impacted the physical structure of the heterotrophic microbial biofilm community. The control treatment biofilm growth displayed a dense layer of bacterial cells with minimal vertical development (Fig. 3). The 2X, 4X, 8X and 12X enrichments showed increasing vertical

development consisting of filamentous growth with interspersed bacterial cells (Fig. 3). This top layer was underlain by dense bacterial growth (Fig. 3).

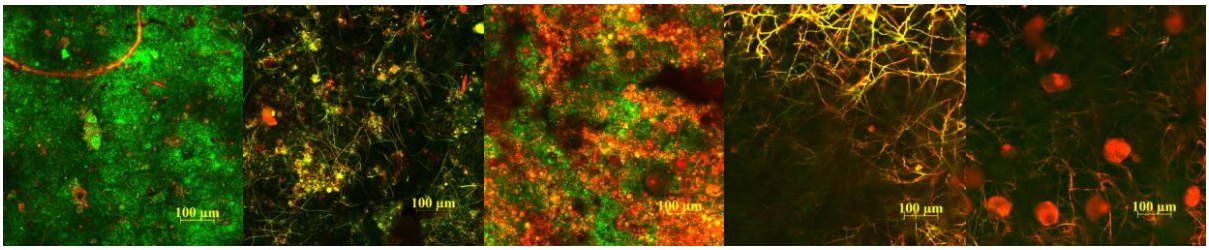


Figure 3: Confocal images from control, 2X, 4X, 8X, and 12X treatments (from left to right).

Effects of Eutrophication on Biofilm Function:

Eutrophication had a positive effect on the areal activities of all five enzymes (Fig. 4, Table1). The absolute activities of the enzymes increased in the order ALPHA, NAG, BETA, LEU, and PHOS, while the relative increases with respect to enzyme activities in the Control treatment increased in almost the reverse order of PHOS, LEU, BETA, ALPHA, and NAG (Fig. 4, Table 1). The enzyme activities increased non-linearly along the eutrophication gradient with disproportionately higher increases found at the 8X and 12X levels of eutrophication (Fig. 4).

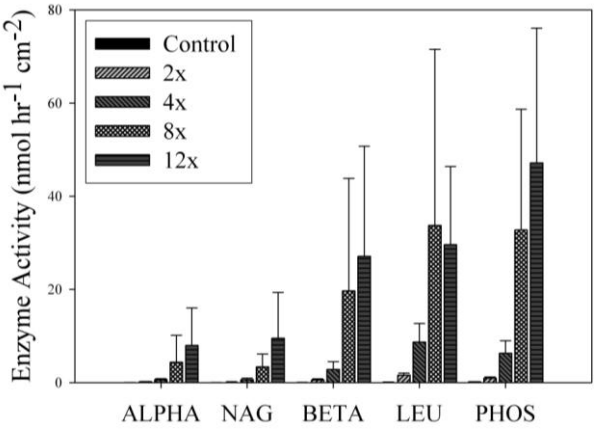


Figure 4: Enzyme activities (nmol hr^{-1} cm^{-2}) for five extracellular enzymes from the enrichment gradient (ordered Control through 12x).

Enzyme	Control	2X	4X	8X	12X
ALPHA	1	41	163	1141	2088
NAG	1	83	491	2798	7924
BETA	1	20	109	768	1058
LEU	1	24	130	508	446
PHOS	1	7	45	235	338

Table 1: Increases in extracellular enzyme activities from levels found in control treatment (Ex. the 2X treatment had 41 times more ALPHA activity than the control treatment).

Effects of Eutrophication on Biofilm Community Diversity and Structure:

General Distribution of Bacterial 16S rRNA Sequences. A total of 2096 high quality partial 16S rRNA gene sequences were obtained. These sequences were distributed between the eutrophication treatments with 413, 441, 430, 419, and 393 sequences from the Control, 2X, 4X, 8X, and 12X treatments respectively.

Rarefaction curves from the four enrichment treatments approached a plateau indicating nearly complete sampling of the diversity in these samples (Fig. 5). However, rarefaction curves from the Control channels did not approach a plateau indicating not all of the diversity in these biofilms was sampled and that these samples had the highest bacterial diversity (Fig. 5). The rarefaction curves from the enrichments indicated diversity decreased in the order: 4X, 8X, 2X, and 12X (Fig. 5).

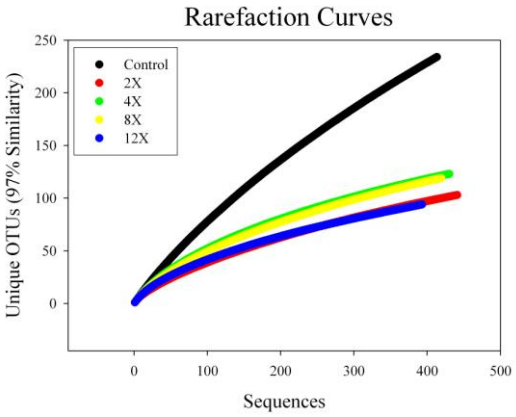


Figure 5: Rarefaction curves for bacterial 16S rRNA genes from the Control, 2X, 4X, 8X, and 12X eutrophication treatments.

Effects of Eutrophication on Biofilm Community Structure. UPGMA clustering of the sequence data in UniFrac (1000 permutations, Un-weighted) revealed ten sample groupings that were well supported by jackknife bootstrap analysis (Fig. 6). The Control, 2X, and 4X samples grouped into three distinct clades, indicating that the community structures of these samples were unique from each other and the 8X and 12X samples (Fig. 6). The 8X and 12X samples grouped together in a fourth clade with mixed grouping of samples among these two treatments (Fig. 6).

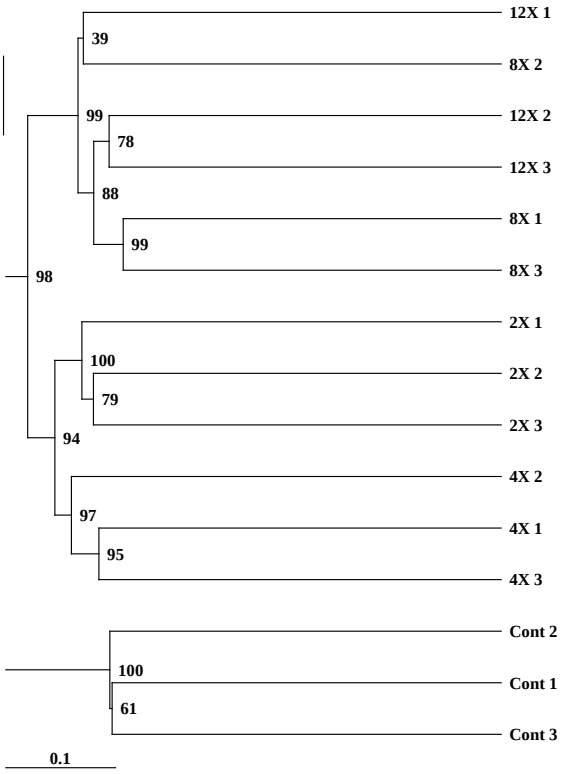


Figure 6: UPGMA clustering of bacterial communities from the Control, 2X, 4X, 8X, and 12X eutrophication treatments with bootstrap values displayed at the nodes.

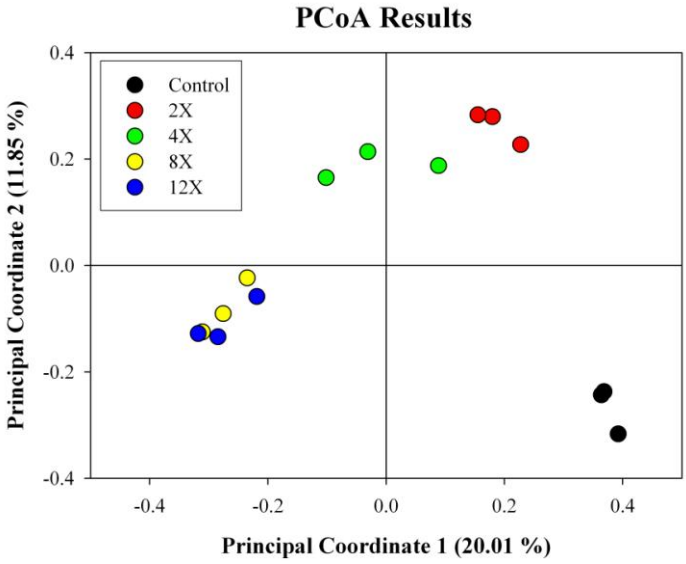


Figure 7: PCoA clustering of bacterial communities from the Control, 2X, 4X, 8X, and 12X eutrophication treatments.

PCoA results for the fifteen samples show relatively tight and distinctive grouping of the Control, 2X, and 4X samples and a grouping that includes samples from the 8X and 12X treatments (Fig. 7). Principal Coordinate 1 explained 20% 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 right to left side of the plot (Fig. 7).

The Presence of Eutrophication Induced Tipping Points:

The AFDM and live cell data indicate the presence of an eutrophication inducted productivity tipping point between a 4X and 8X enrichment (Fig. 2). Both variables responded slightly to the 2X and 4X enrichments and very strongly to 8X and 12X enrichments. Biofilm function also appears to have an eutrophication threshold as the response of extracellular enzyme activity to enrichment was moderate up to the 4X enrichment level and then increased dramatically at the 8X and 12X enrichment levels (Fig. 4). The effects of eutrophication on biofilm community structure also appears to be non-linear, with a gradual shift in community structure between samples from the Control, 2X, and 4X treatments and clustering of samples from the 8X and 12X communities indicating further enrichment has no additional affect on community structure (Fig. 6 and Fig. 7).

Discussion:

The first goal of this study was to determine how resource supply/eutrophication affects stream biofilm community productivity. It was hypothesized that because biofilm productivity has been shown to be responsive to nutrient availability (Costerton et al. 1995), eutrophication would increase biofilm biomass and the relative abundance of live cells. Our AFDM data and the relative abundance of live cells supported this hypothesis and indicate that stream biofilms responded dramatically to increased resource supply, particularly when resource levels are four to eight times greater than background levels.

The second goal of this study was to determine if changes in productivity affect biofilm community diversity and structure. 16S rRNA gene sequence data from this study indicated that the Control treatment channels had the highest diversity and that nutrient additions significantly decreased the diversity of the biofilm communities. These findings are unexpected because they appear to contradict the findings of several reviews of plant and animal studies that investigated the effects of productivity on biodiversity (Waide et al. 1999, Mittelbach et al. 2001, Evans et al. 2005). These reviews indicate that for hundreds of plant and animal communities the most common productivity-diversity relationships are unimodal at local scales and monotonically increasing at regional scales. Numerous mechanisms have been proposed to explain these relationships, however, the most common explanation states that in low productivity/available energy systems, insufficient resources exist to support many species at viable population levels. As productivity increases, the number of viable populations and the diversity increase. At local scales when productivity levels are high, interspecific competition increases, driving some species to extinction (Rajaniemi 2003). Additionally, increased productivity/available energy is thought to increase the abundance of rare resources, opening new niches for exploitation by rare species (Evans et al. 2005). If these same patterns and processes were occurring in the biofilms we studied, we would have expected to see an increase in diversity at intermediate levels of eutrophication (2X and 4X treatments) and a decrease in diversity at the high levels of eutrophication. This seemed particularly likely for biofilm communities due to the increased number of physical and chemical niches that form as biofilms mature and thicken. Instead, it appears that some member of the biofilm communities were able to out compete and drive to extinction numerous members of the biofilm population, ultimately leading to a decrease in biofilm community diversity.

The third goal of this study was to determine if changes in community diversity or structure affect biofilm function. The ecological community has been debating the effects of biodiversity on ecosystem functioning for several decades. This argument is driven by a desire to understand and predict how current human induced loss of diversity, in what some consider a mass extinction event (Woodruff 2001), will alter the ability of ecosystems to provide services such as carbon sequestration and nutrient retention and processing. This debate has fueled an explosion of research, and while uncertainty remains about such issues as the relative importance of species versus functional diversity, a review of many relevant studies found a generally positive relationship between diversity and ecosystem function for macro-organisms (Loreau et al. 2001, Hooper et al. 2005). Few studies have investigated diversity-function relationships in microbial communities due to logistical difficulties; however, some relevant data exist. Three soil studies used toxic substances, copper, mercury, benzene, and chloroform, to experimentally decrease soil microbial diversity. They found broad scale parameters that are influenced by the entire microbial community such as respiration and decomposition rates were unchanged. However, more specific parameters, which involve a small subset of the community such as nitrification, denitrification and methane oxidation, decreased as diversity decreased (Griffiths et al. 2000, Muller et al. 2002, Girvan et al. 2005). A single aquatic study has shown a strong positive relationship between bacterial diversity and community respiration rates, indicating in some systems broad scale functions may also be affected by changes in diversity (Bell et al. 2005). In the biofilm communities we investigated in this study, the decreases in diversity that resulted from nutrient additions did not translate into a decreased ability to process large organic molecules as measured by extracellular enzyme activity. This response may be due to the physical changes that occurred in the

biofilms as a result of enrichment. Increased biofilm thickness may protect extracellular enzymes from being washed out of the system by downstream flow. Microbes may also secrete higher quantities of enzymes in this protected environment where their investment is protected.

The final goal of this study was to determine if enrichment thresholds exist that if crossed, result in rapid changes in community structure and function. Metazoan communities have been shown to respond to changing environmental conditions by following one of three general patterns: 1) a gradual, smooth response, 2) a gradual response until a tipping point is reached initiating rapid change, and 3) a gradual response followed by a shift to an alternative stable state (Scheffer et al. 2001, Scheffer and Carpenter 2003), however, very little information exists regarding the response of microbial biofilms to disturbance. Our data indicate biofilm communities respond nonlinearly to eutrophication with a productivity/function tipping point occurring between 4X and 8X enrichments that results in a dramatic increase in productivity and function. Our data also indicate that any level of enrichment dramatically decreases diversity in stream biofilm communities and that a gradual shift in community structure occurs as streams are enriched.

Conclusions:

Stream biofilm communities play a significant role in processing nutrient and organic matter inputs to streams. As streams are enriched with nutrients and dissolved organic carbon the community diversity and structure of these biofilm communities as well as their ability to perform ecosystem functions undergo significant changes. These responses do not follow the patterns expected from the extrapolation of results from studies of metazoan communities, indicating that the ecology of microbial biofilm communities may be unique.

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