

## ABSTRACT

Title of Document: ALTERATIONS TO HEADWATER STREAM  
MICROBIAL COMMUNITIES AND CARBON  
CYCLING IN RESPONSE TO  
ENVIRONMENTAL CHANGE

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Organic carbon, principally as dissolved organic matter (DOM), is a fundamental energy source that powers microbial metabolism and shapes food webs in stream ecosystems. The community structure and metabolic activity of stream microbes are significantly impacted by the quantity and quality (i.e. molecular structure) of organic matter resources. Much of the organic matter in headwater streams originates on landscapes. Thus, external inputs of terrestrial organic carbon shape microbial community structure and, subsequently, food webs of headwater streams. Despite the recognized importance of DOM, there is limited understanding of how stream organic matter resources and bacterial community structure respond to watershed urbanization.

I studied DOM quantity and quality, microbial heterotrophic function, and bacterial community composition along a gradient of watershed urbanization in headwater streams of the Parkers Creek watershed (Coastal Plain, Maryland, USA). In Chapter 1, I found that watershed impervious cover was significantly related to stream water DOM

composition: increasing impervious cover was associated with decreased amounts of natural humic-like DOM and enriched amounts of anthropogenic fulvic acid-like and protein-like DOM. The DOM found in urbanized streams was more bioavailable, but only during spring and summer experiments. I report in Chapter 2 that microbial heterotrophic enzyme production was not strongly related to urbanization. Instead, enzyme levels were most strongly related to temperature and natural groundwater chemical gradients. I show in Chapter 3 that bacterial community composition and co-occurrence patterns also changed significantly in response to increasing urbanization, becoming more dominated by primary producers common to eutrophic waters.

I conclude from my research that watershed urbanization fundamentally alters microbial communities and carbon cycling in headwater streams. This urbanized material is more readily metabolized by microbial communities, but only during warmer months.

Increased biodegradation of DOM in warm seasons was related to greater microbial enzyme activity, which generally responds positively to increasing temperature. Thus, rising temperatures with climate change and urbanization combined with altered organic matter content are predicted to result in greater CO<sub>2</sub> evasion from urbanized streams.

ALTERATIONS TO HEADWATER STREAM MICROBIAL COMMUNITIES AND  
CARBON CYCLING IN RESPONSE TO ENVIRONMENTAL CHANGE

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Dissertation submitted to the Faculty of the Graduate School of the  
University of Maryland, College Park, in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2015

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## Preface

This dissertation consists of an introduction, three research chapters, and a summary and conclusions section. All research chapters are presented in manuscript form with introduction, methods, results, and discussion. Tables, figures, and captions occur at the conclusion of each chapter. A single literature cited section occurs at the end for references made throughout the dissertation.

## Acknowledgements

I would first like to thank my advisor, Margaret Palmer, for giving me the opportunity to pursue this research. Margaret has provided me with amazing guidance and support while giving me the intellectual freedom to develop new ideas independently. The result was a very rewarding experience in graduate school, for which I'm very grateful. I would also like to thank the members of my committee, Lora Harris, Andrew Heyes, Bruce James, and Karen Prestegaard for their guidance and insight over the years. Their input has certainly made this a better dissertation.

I am extremely grateful to all of the members of the Palmer Lab Group including Alec Armstrong, Maíra Bezerra, Laura Craig, Steve Epting, Rosemary Fanelli, Catherine Febria, Kelly Hondula, Ben Koch, Lauren Koenig, Brian Laub, and Owen McDonough, who have been so helpful and supportive over the years. I am also grateful to all the collaborators who have provided laboratory resources, technical insight, field assistance, and other support including Solange Filoso, James Gantt, Dale Garner, Megan Lang, Greg McCarty, Tom Miller, Carys Mitchelmore, Henry and Will Nussman, Kristin Politano, Kenny Rager, Johan Schijf, Chris Swan, Lisa Wainger, Michael Williams, and Stephanie Yarwood.

This research was supported by a number of sources including a Doctoral Dissertation Improvement Grant from the National Science Foundation (Award No. DEB-1210516), a Darwin Fellowship from the Behavior, Ecology, Evolution, and Systematics Graduate Program at the University of Maryland (UMD), a summer research fellowship from the

UMD Graduate School, and grants awarded to Margaret Palmer from NOAA (Grant No. NA10OAR431220), the Environmental Protection Agency (Grant No. GS-10F-0502N), and the National Science Foundation (Grant No. DBI-1052875).

Lastly, I would like to thank my family, friends, and Lindsay Bloch, who have all been such important sources of help and support over the last few years.

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## Introduction

Organic carbon, principally as dissolved organic matter (DOM; (Wetzel, 1992), is a fundamental energy source that powers microbial metabolism and shapes food webs in stream ecosystems (Hall and Meyer, 1998; Battin et al., 2008; Gücker et al., 2011).

Microbial heterotrophs metabolize organic matter and primary producers fix new autochthonous carbon. In turn, organic matter quantity and quality (*i.e.*, molecular structure) broadly shape microbial community structure (Judd et al., 2006, 2007) and ecosystem function (Hall and Meyer, 1998; Bernhardt and Likens, 2002; Sviridov et al., 2011; Newcomer et al., 2012). Much of the organic matter in headwater streams originates on landscapes (Aitkenhead-Peterson et al., 2003; Battin et al., 2008). Thus, external inputs of terrestrial organic carbon shape microbial community structure and, subsequently, food webs of headwater streams.

Freshwaters were once thought to be unimportant to the global carbon cycle, but there is growing recognition that freshwater systems transport a significant amount of carbon (Cole et al., 2007; Tranvik et al., 2009; Raymond et al., 2013). These water bodies act as more than just conduits for the transport of carbon from landscapes to the sea. Uptake of organic matter, primarily by microbes, results in substantial retention of organic matter. Much of the organic matter captured by microbes is ultimately respired and released from streams as carbon dioxide. Increased scrutiny of organic matter processing in inland waters has continued to reveal a larger role for the importance of carbon processing in freshwaters. Estimates of carbon dioxide evasion from inland waters have risen quickly

in a short amount of time from 0.75 Pg C y<sup>-1</sup> (Cole et al., 2007) to 1.4 Pg C y<sup>-1</sup> (Tranvik et al., 2009) to 1.8 Pg C y<sup>-1</sup> (Raymond et al., 2013). Estimates are improving, but there is still much ambiguity about the amount and ultimate fate of carbon in freshwater systems as a whole. Estimates of the contribution of headwater streams to carbon fluxes are particularly uncertain because of the large number and high heterogeneity of these systems (Cole et al., 2007).

Even as scientists are quantifying the freshwater carbon cycle, many aspects of the global carbon cycle are being perturbed by global environmental change. Thawing of permafrosts are releasing into waterways stores of organic matter that have remained in place for thousands of years (Cory et al., 2013; Abbott et al., 2014; Cory et al., 2014). Freshwater boreal systems around the world are becoming more humic – a development called ‘browning’ – a process likely driven by altered hydrology from climate change and decreasing intensity of acid rain (Monteith et al., 2007; Haaland et al., 2010; Weyhenmeyer et al., 2014). Agricultural land use increases fluxes of labile fulvic acids from watersheds and stimulates autochthonous organic matter production by microbes in streams (Wilson and Xenopoulos, 2008; Williams et al., 2010; Stanley et al., 2012). Despite broad recognition of human-induced changes to the carbon cycle, surprisingly little research has been devoted to the study of landscape urbanization. Still, there is ample reason to expect that stream catchment urbanization strongly influences organic carbon and microbial community structure and function.



How urbanization likely impacts microbial communities and organic matter cycling begins on land. Urbanization depletes soil organic matter content through earth moving activities and similar forms of disturbance (Pizzuto et al., 2000; Lorenz and Lal, 2009). Impervious cover, ground compaction, and loss of vegetation lead to increased overland flow in urbanized systems (Dunne and Leopold, 1978; Arnold and Gibbons, 1996). Heightened surface runoff during development results in disproportionately high levels of soil organic matter loss, due to the low density of organic matter compared to other soil fractions (Lal, 2003). Impervious cover also blocks water infiltration into underlying material (Lorenz and Lal, 2009), which prevents transport of soil microbes and humic substance-rich organic matter from soil to stream.

In addition to sealing off terrestrial organic matter and microbes from streams, urbanization also enhances inputs of other materials into streams. These watersheds are sources of microbes, and nutrients, and, potentially, organic matter that alter stream environments. Septic systems (Steffy and Kilham, 2004), leaking sewers (Kaushal and Belt, 2012), petroleum products (McElmurry et al., 2013), and stormwater management ponds (Williams et al., 2011) are known or suspected to be sources of microbially produced organic matter to streams.

The quality of organic matter inputs to a stream is important because it can strongly alter system functioning. Rapidly growing heterotrophic microbial communities quickly metabolize certain bioavailable (i.e. microbially biodegradable) fractions found in organic matter (Hudson et al., 2008) from sources such as wastewater treatment plants

(Stedmon and Markager, 2005; Gücker et al., 2011) and agricultural watersheds (Wiegner and Seitzinger, 2004; Wilson and Xenopoulos, 2008; Williams et al., 2010; Juckers et al., 2013). Urban streams are sites of accelerated biogeochemical transformations in response to high levels of nutrients, decreased riparian canopy cover, and an altered microbial community (Sivirichi et al., 2011; Kaushal and Belt, 2012). This led me to hypothesize that urbanized streams would contain increased levels of bioavailable DOM, as has been seen in other human-impacted freshwaters.

For this dissertation, I observed the effects of watershed urbanization on 1) organic matter resources; 2) microbial heterotrophic function; and 3) microbial community structure in headwater streams. To do this, I studied a series of headwater streams spanning a gradient of urbanization within the Parkers Creek watershed; a Coastal Plain system in Maryland, USA. Research was conducted in a single watershed to eliminate variability due to climate and geological differences. Parkers Creek served as an ideal study system because of the relatively high proportion of forested cover, approximately 72%, across the watershed.

I examined how stream organic matter cycling and microbial community structure and heterotrophic metabolic function respond to watershed urbanization. I applied observational field measurements of stream organic matter quantity and quality, nutrient levels, and bacterial community composition. I coupled field measurements with laboratory analysis of microbial extracellular enzyme activity and DOM bioavailability. I

present below a brief outline of the chapters presented here and the major findings of this research.

**Chapter 1: Dissolved organic matter quality and bioavailability changes across an urbanization gradient in headwater streams.**

I first investigated the organic matter resource that forms the base of the food web in most stream ecosystems, dissolved organic matter (DOM). I studied the changes to stream DOM quantity, quality and microbial bioavailability across a gradient of catchment urbanization, as measured by watershed impervious cover. The impacts measured were non-point source in nature as none of the streams had documented or observed point source discharges. I measured DOM quality using fluorescence excitation-emission matrices (EEMs) coupled with parallel factor analysis (PARAFAC). I assessed DOM bioavailability using biodegradable dissolved organic carbon (BDOC) incubations.

Watershed impervious cover was significantly related to stream DOM composition: increasing impervious cover was associated with decreasing amounts of natural humic-like DOM and enriched amounts of anthropogenic fulvic acid-like and protein-like DOM. Bioavailability of DOM was significantly related to decreasing proportions of humic-like DOM and increasing proportions of protein-like DOM, but only during spring and summer months. As a result, microbial bioavailability of DOM was greater in urbanized than forested streams, but only during warm seasons. Increased bioavailability during spring and summer experiments was associated with elevated extracellular enzyme

activity of the initial microbial community supplied to samples during BDOC incubations. These findings indicate that changes in stream DOM quality due to watershed urbanization most strongly impact stream ecosystem metabolism under warmer conditions. Thus, the impacts of watershed impervious cover and increasing temperatures as a result of climate change may interact to amplify release of CO<sub>2</sub> from headwater streams impacted by urbanization.

## **Chapter 2: Stream water temperature and groundwater chemistry impact microbial extracellular enzyme activity in headwater streams.**

To identify the factors governing microbial organic matter metabolism, I measured stream microbial enzyme activity across space and time. Microbes release substrate-specific extracellular enzymes to break down large organic molecules for subsequent uptake. This is the rate-limiting step controlling microbial heterotrophic metabolism in environmental systems. I seasonally measured microbial production of seven extracellular enzymes in Parkers Creek headwater streams. Temperature was a central factor governing levels of microbial enzymes, with some enzymes exhibiting non-linear temperature dependence. A north-south trend in enzyme activity was related to a shift in source water conductivity and ion chemistry. Changes to source water chemistry were linked to natural groundwater chemical gradients, suggesting that microbial metabolism in streams is constrained by underlying geology. In contrast to DOM quality, enzyme activity was not strongly related to watershed urbanization.

### **Chapter 3: Analysis of headwater stream bacterial diversity reveals significant influence of urbanization and habitat on community structure.**

Bacterial community composition is recognized to shift in response to urbanization, but the environmental conditions and specific taxonomic changes related to this shift are not currently known. Here, I measured differences in bacterial community structure across stream site (urbanized, forested) and habitat type (water column, streambed sediments). I measured bacterial community composition with 16S rDNA sequences collected from eleven stream sites from the Parkers Creek watershed. I found that alpha diversity in sediment was slightly lower compared to the water column across sites. Sediment and water column bacterial community composition were distinct across all sites studied. I found a significant shift in community composition in response to watershed urbanization in both stream water column and sediment samples. Bacterial community composition in sediments was more strongly related to the environmental factors measured than water column community composition. In contrast to overall community structure, microbial co-occurrence networks were more tightly linked to watershed urbanization than sample habitat type. Network analysis revealed that urbanized microbial networks were more aggregated – indicated by higher network modularity – than forested networks; a characteristic that can be indicative of microbial responses to disturbance. Thus, both bacterial community composition and how bacterial communities interact are strongly tied to environmental characteristics related to watershed urbanization.

I conclude from my research that urbanized landscapes lead to reduced amounts of natural, humic organic matter and increased quantities of microbially produced organic

matter. This urbanized material is more readily metabolized by microbial communities in summer months, which was related to greater microbial enzyme activity. Thus, rising temperatures with climate change and urbanization combined with altered organic matter content are predicted to result in greater CO<sub>2</sub> evasion from streams. Bacterial community composition shifted significantly with response to urbanization; becoming more dominated by primary producers common to eutrophic waters.

# Chapter 1: Dissolved Organic Matter Quality and Bioavailability Changes across an Urbanization Gradient in Headwater Streams

## **Introduction**

Freshwater ecosystems bury or return to the atmosphere an estimated 1.0 Pg C y<sup>-1</sup> (Cole et al., 2007), with rivers transporting approximately 0.2 Pg C y<sup>-1</sup> as dissolved organic matter (DOM; (Butman et al., 2012). Most of the DOM present in headwaters originates in terrestrial ecosystems (Aitkenhead-Peterson et al., 2003). Situated at the origins of river networks, headwater streams represent the transition from terrestrial to aquatic and play an integral role in global carbon cycling as sites of significant organic carbon processing and transport (Gomi et al., 2002; Wipfli et al., 2007; Battin et al., 2008). Processing by the microbial community is responsible for a large fraction of total stream metabolism in headwater channels and is driven by the quantity and quality (i.e., molecular structure) of DOM (Judd et al., 2006; Fellman et al., 2008; Cory and Kaplan, 2012). Microbial processing can determine whether headwater stream organic carbon is delivered downstream, enters stream food webs, or is metabolized and released from streams in gaseous form (Battin et al., 2008; Wilson and Xenopoulos, 2008). Changes in DOM quality can impact ecosystems through alterations of DOM uptake (Sivirichi et al., 2011), food web dynamics (Hall and Meyer, 1998; Gücker et al., 2011), and, coupled biogeochemical cycles including nitrogen uptake (Bernhardt and Likens, 2002) and denitrification (Newcomer et al., 2012).

Stream DOM quantity and quality are related to watershed attributes including soil type (Fellman et al., 2008), wetland coverage (Richardson et al., 2009; Williams et al., 2010), agricultural land use (Wilson and Xenopoulos, 2008; Williams et al., 2010; Stanley et al., 2012), and urban point-source inputs such as from wastewater treatment facilities (Stedmon et al., 2003; Baker and Spencer, 2004; Sickman et al., 2007; Gücker et al., 2011). Typically this material is highly bioavailable DOM with fluorescence signatures that resemble microbially fixed fulvic acids and protein-like material (Stedmon et al., 2003; Stedmon and Markager, 2005; Gücker et al., 2011). In contrast, non-point source inputs of anthropogenically altered carbon from urban landscapes are poorly understood, particularly in terms of the contribution to DOM quality. This knowledge gap is noteworthy given recent findings that urbanization can increase (Gücker et al., 2011; Imberger et al., 2014) and alter (Newcomer et al., 2012) labile autochthonous particulate organic matter inputs to streams.

Runoff from asphalt surfaces on roads and parking lots as well as soils and turf grass in urban open areas may contribute substantial amounts of DOM to receiving waters (Aitkenhead-Peterson et al., 2003; Sickman et al., 2007) and this DOM may be lower in the recalcitrant fractions that characterize forest- and wetland-dominated landscapes. Increased DOM bioavailability in urbanized streams was reported in one study but the source of this altered DOM was uncertain (Imberger et al., 2014). Organic matter from urban surfaces could have been the source since a recent study of runoff from a variety of urbanized landscapes observed DOM that was lower in molecular weight and aromaticity but higher in hydrophobicity compared to forested run-off (McElmurry et al., 2013).



Given increasing urbanization (Baum et al., 2013), it is important to advance my understanding of the ecological impacts of diffuse, non-point source inputs of DOM to streams.

My goal was to quantify changes in DOM quantity, composition, and bioavailability in response to increasing non-point source inputs to streams associated with a gradient of catchment urbanization. I studied eight Coastal Plain streams of Maryland, USA that ranged from completely forested to highly urbanized (>40% impervious cover).

Anthropogenic impacts in urbanized watersheds include suburban homes, retail space, parking lots, and stormwater management ponds, but no point-source inputs. I

hypothesized that compared to forested or less urbanized streams, DOM in those streams most impacted by urbanization would be: 1) more aromatic and autochthonous in nature; and 2) more readily used by microbial communities. I further hypothesized that: 3) microbial use of the DOM would not only depend on its composition but on the heterotrophic activity of the microbial community.

## **Methods**

*Study Sites.* Samples were collected from eight first-order headwater streams located in the Parkers Creek watershed in the Coastal Plain of Maryland, USA (Figures 1.1 and 1.2). The Parkers Creek watershed is approximately 3,107 hectares in size and is largely composed of alluvial sediments. Five of study streams were categorized forested and have less than 5% impervious cover. Three study streams are located in urbanized watersheds and have at least 10% impervious cover (Table 1.1).

*Stream DOM Quantity and Quality.* A total of 143 samples were collected on 20 occasions during baseflow conditions from March 2011 through February 2013. For the purposes of this study, baseflow conditions were defined as any period more than 48 hours after a rain event. Sites F1, F4, and F5 did not flow during some sampling events that occurred in summer months (July-September), reducing the total number of samples collected. Samples were collected approximately monthly, all within 36 hours of each other. There were no precipitation events between the start and finish of any sampling event. Samples were filtered in the field using Whatman GF/F filters with a nominal pore size of 0.7  $\mu\text{m}$  that were pre-combusted at 450°C. Water samples for DOM analysis were collected in acid-washed, amber glass bottles that were pre-combusted at 450°C; other water samples were collected in acid-washed HDPE bottles. All samples were transported to the laboratory on ice and stored at 4°C prior to further processing. Dissolved Organic Carbon (DOC), as non-purgeable organic carbon, and total dissolved nitrogen (TDN) concentrations were determined using a Shimadzu TOC-vCPH total organic carbon analyzer with attached TNM-1 total nitrogen analyzer.

DOM fluorescence spectroscopy was conducted following established methods (Andersson and Bro, 2000; Stedmon et al., 2003; Stedmon and Bro, 2008). Fluorescence excitation-emission matrices were acquired using a Horiba Fluoromax-4 spectrofluorometer. To obtain fluorescence excitation-emission matrices (EEMs), samples were excited from 250 to 450 nm at 5 nm increments, and emissions were measured at each excitation wavelength from 300 to 550 nm at 2 nm increments. Blank

EEMs were collected routinely using Nanopure water. The mean area under the Raman peak at 350 nm of the Nanopure blanks collected during each fluorescence analysis run was used to normalize EEMs. Fluorescence EEMs for each sample were corrected for machine bias, inner-filter effects, and Raman scatter (Cory et al., 2010). UV/Vis absorbance spectra for each sample were collected from 200 to 800 nm at 1 nm increments using a Thermo Scientific Evolution 60 spectrophotometer. Specific ultraviolet absorbance at 254 nm ( $SUVA_{254}$ ) increases with DOM aromatic content and was measured by taking the absorbance of a DOM sample at 254 nm and dividing it by the DOC concentration (Weishaar et al., 2003). Iron interference of absorbance at 254 nm was identified in some samples. For a subset of samples collected November 2011, April 2012, August 2012, November 2012, and February 2013, total iron was determined using the Hach FerroVer method. Iron concentrations were used to correct absorbance at 254 nm using a previously developed relationship:  $A_{254\text{-corrected}} = A_{254\text{-measured}} - 0.0687 * [Fe^{3+}]$  (O'Donnell et al., 2012).

To obtain DOM quality metrics from fluorescence EEMs, PARAFAC analysis was conducted using the DOMFluor Toolbox (Andersson and Bro, 2000) in Matlab version R2013a. An EEM-PARAFAC model based on 417 EEMs from Maryland Coastal Plain streams, including the 143 used in this study, was validated using split-half analysis (Stedmon and Bro, 2008). Five fractions of DOM were validated using PARAFAC analysis and the form of DOM each component likely represents was identified from previous studies (Table 1.2; Figure 1.3). Two fluorescence indices, the fluorescence index (FI) (McKnight et al., 2001) and the humification index (HIX) (Zsolnay et al., 1999),

were also used to describe DOM composition. Fluorescence index values were computed as the ratio of emission intensities at 450 and 500 nm at an excitation wavelength of 370 nm (McKnight et al., 2001). FI is indicative of DOM source: higher values indicate increased autochthonous DOM while lower values are indicative of allochthonous DOM. HIX values were computed based on existing methods (Zsolnay et al., 1999; Plaza et al., 2009; Williams et al., 2010) as the ratio of the area of the emission spectrum at 435 to 480 nm to the emission area from 300 to 445 nm at an excitation wavelength of 255 nm. Increasing HIX values are indicative of more humic, structurally complex DOM.

*Microbial Utilization of DOM.* To determine DOM bioavailability, bioavailable dissolved organic carbon (BDOC) bioassays were conducted following established methods (Fellman et al., 2008) in November 2011, April 2012, August 2012, November 2012, and February 2013, resulting in 36 samples. Bioassays measure the loss of DOC through net bacterial production and respiration over the course of 28-day incubations (McDowell et al., 2006). Water samples were filtered to 0.22  $\mu\text{m}$  using Millipore polycarbonate membrane filters within 24 hours of collection from the field. Microbial inoculum was obtained by collecting sediment and water from site P1 on the main stem of Parkers Creek (Table 1.1) contemporaneously with other sample collection.

Approximately 100 g of sediment were combined with 800 mL of stream water and this slurry was allowed to incubate overnight in the laboratory, after which the mixture was filtered using 0.7  $\mu\text{m}$  pre-combusted glass fiber filters. A 48 mL aliquot of filtrate and 2 mL of microbial inoculum were added to a pre-combusted amber glass bottle, which was then thoroughly mixed. For each sample, six replicate incubations were prepared; three

replicates for each sample were immediately filtered to 0.22  $\mu\text{m}$  to serve as Day 0 samples. The remaining bottles were incubated for 28 days in the dark at 20°C with caps vented to allow airflow. Samples were regularly agitated to prevent anoxia. After 28 days, samples were filtered to 0.22  $\mu\text{m}$ , and were analyzed for DOC concentration. Percent BDOC was calculated as the percent DOC lost over the course of the 28-day incubation. To account for potential microbial production of DOM, triplicate control vials that contained Nanopure water in place of stream water were also incubated with microbial inoculum on each sampling date. Paired t-tests that compared the DOC concentrations of 0- and 28-day control samples did not detect any significant changes.

Incubations were conducted without amending samples with nitrogen and phosphorus to prevent nutrient limitation, as has been done elsewhere (McDowell et al., 2006). This was done to allow microbial lability of DOM to be analyzed in the context of environmental nutrient levels. To account for potential nutrient limitation, nitrate, ammonium, and orthophosphate concentrations were determined using a Lachat QuikChem 8500 Series 2 flow injection analyzer. Molar nitrogen to phosphorus (N:P) ratios were computed as the ratio of dissolved inorganic nitrogen to orthophosphate-phosphorus.

*Microbial Heterotrophic Enzyme Production.* Extracellular enzyme activities (EEA) measure the forms of DOM utilized by stream microbial communities. These enzymes are produced based on the metabolic demands of microbes and the resources available in the environment (Hill et al., 2012). Whole water samples were collected from site P1 contemporaneously with the inoculum used for BDOC assays and were processed for

EEA within 24 hours of collection. Microplate EEA studies were conducted based on existing methods (Sinsabaugh, 1997; Findlay, Stuart, 2007). Activity of extracellular enzymes was measured by assessing the degradation rates of fluorescently labeled model substrates by stream water. Substrates 4-methylumbelliferone butyrate (MUB)-acetate, 4-MUB-phosphate, and L-Leucine 7-amido-methyl-coumarin were used to measure the activity of esterase, alkaline phosphatase, and leucine aminopeptidase, respectively. The 4-MUB-acetate and 4-MUB-phosphate buffers were prepared in autoclaved 5 mM bicarbonate buffer, L-Leucine 7-amido-methyl-coumarin was prepared in autoclaved Nanopure water. Substrates were refrigerated and stirred continuously for 24 hours preceding the experiment to ensure dilution. Equal amounts of stream water and substrate (150  $\mu$ l each, 300  $\mu$ l total volume) were combined in black 96-well plates resulting in a final substrate concentration of 500  $\mu$ M. Each sample was incubated with each substrate in four replicate wells. Each microplate also included replicate stream water, buffer, and substrate controls. Microplates were incubated in the dark at 20°C for four hours and were measured at regular intervals using a Molecular Devices SpectraMAX Gemini XPS plate-reading spectrofluorometer. Standard curves were developed using MUB and coumarin standards. At the conclusion of the experiment photoquenching by samples was measured by spiking buffer and stream water controls with 50  $\mu$ L of 1  $\mu$ M standard. All standards and substrates were obtained from Sigma-Aldrich.

*Landscape Analysis.* A digital elevation model (DEM) of the Parkers Creek watershed was generated from Light Detection and Ranging (LiDAR) data collected in March 2011 and provided by Calvert County, MD Government. Field site watersheds were extracted

from the DEM. Impervious, forested, and agricultural land cover were manually delineated using orthophotography collected in March 2011 and provided by Calvert County, MD Government. Septic system geospatial data were obtained from Maryland Department of the Environment reports (Tetra Tech, 2011). All landscape analysis was conducted using ArcGIS 10.1.

*Data Analysis.* Repeated-measures analysis of variance (ANOVA), analysis of covariance (ANCOVA), and linear regressions were carried out in SAS 9.3 using the *Proc Mixed* package. Normality of ANOVA and ANCOVA variables was evaluated by assessing residuals. Variables that deviated substantially from normality were transformed by taking the logarithm of the variable plus one. Compound symmetry covariance structures were assumed for all ANCOVA analysis. Multiple mean comparisons for repeated-measures ANOVA were conducted using Tukey's Honestly Significant Difference (HSD) test. For bioavailability analysis, sampling dates were broken into two groups, "Fall/Winter" and "Spring/Summer." A seasonal term and interaction were included for all statistical analyses of bioavailability data. Principal component analysis (PCA) was conducted using the *princomp* function in R version 3.0.2. Normality of all variables used for PCA was assessed with histograms and linearity was assessed with bivariate scatterplots. Prior to analysis, an alpha level of 0.05 was set for all analyses.

## Results and Discussion

*DOM Quantity and Quality across Urbanization Gradient.* Overall DOM quality across sites was evaluated using principal component analysis (PCA) of the five validated PARAFAC components identified distinct differences in DOM composition between forested and urbanized streams, as indicated by a plot of the first two PCA components (Figure 1.4). Forested catchments were characterized by PARAFAC components C1 and C2 and percent fluorescence of both components was positively related to log-transformed percent watershed impervious cover (Figure 1.5 a and b; Table 1.3). Previous studies have linked PARAFAC components similar to C1 and C2 to terrestrial sources of organic matter (Stedmon et al., 2003; Stedmon and Markager, 2005; Osburn et al., 2012) with C2 reflecting recalcitrant, terrestrial humic substances (Stedmon and Markager, 2005; Lutz et al., 2012) and C1 more photolabile sources associated with streams in forested (Stedmon et al., 2003; Stedmon and Markager, 2005) and agricultural (Osburn et al., 2012) watersheds. This is consistent with an interpretation that the DOM found in Parkers Creek forested headwater streams was represented by compounds originating from forest soils and higher plants.

PARAFAC components C3 and C5 were more prevalent in urbanized sites (Figure 1.4). Percent fluorescence of component C3 and log-transformed percent fluorescence of component C5 were both positively related to log-transformed watershed impervious cover (Figure 1.5 c and d; Table 1.3). Component C3 most likely represents anthropogenic humic acids that are microbially produced that have been linked to agricultural land use and point sources like wastewater effluent (Stedmon and Markager,



2005) but these impacts are not characteristic of the streams studied. The EEM modeled for component C5 is tyrosine-like (Fellman et al., 2008) and hence likely a product of aquatic carbon fixation and relatively labile.

The DOM absorbance metrics provided further evidence of a DOM quality shift with increasing impervious cover. Aromaticity of DOM was measured via  $SUVA_{254}$ , which was negatively correlated with log-transformed impervious cover (Figure 1.3h). Lower DOM molecular weight and aromaticity in the urban streams provided verification that this material is of recent, microbial origin. A significant positive relationship between catchment DOC yield and percent watershed impervious cover was found (Figure 1.5g; Table 1.3). By contrast, neither DOC flux nor concentration across sites was significantly related to catchment impervious cover (Table 1.3), suggesting that increased DOC yield was related to altered hydrology in urbanized catchments.

*Possible Urban Sources of DOM.* The measured changes in DOM quality (lower molecular weight and aromaticity) reported here support Hypothesis 1, that the urbanized streams have become enriched in the same forms of DOM found in other anthropogenically impacted waters (e.g., agricultural streams and wastewater effluent (Wilson and Xenopoulos, 2008; Williams et al., 2010; Stedmon et al., 2003). The ecosystem processes driving these changes were less clear. DOM quality across the three urbanized streams was very similar, yet the nature of the anthropogenic impacts was more variable across the urbanized watersheds. The watershed of site U1 is 44.0% impervious and is dominated by commercial space and asphalt parking lots. By contrast,

the primary human impacts in watersheds U2 and U3 were residential housing and associated roadways. Site U3 receives water directly from a stormwater management pond; no other watersheds included such a structure.

One possible explanation for the altered DOM composition is increased primary production within and around urbanized streams. Fluorescence index values and log-transformed percent watershed impervious cover were positively correlated (Figure 1.5e; Table 1.3). Increasing FI values indicate greater prevalence of aquatically fixed DOM in urbanized streams (McKnight et al., 2001), providing evidence of the autochthonous nature of urbanized DOM. Total dissolved nitrogen (TDN) yields and concentrations were strongly positively related to percent catchment imperviousness (Figure 1.5h; Table 1.3) which would enhance autochthonous production in streams (Bernot et al., 2010). Primary production may also have been stimulated by lower canopy cover in urbanized streams (Bernot et al., 2010).

There are also potential non-point sources of anthropogenic DOM in urban landscapes. There are few septic systems within any of the study watersheds (Table 1.1), which precluded examination of this factor. While the urbanized watersheds do not contain farmland or point source effluent inputs, all three urbanized watersheds do contain sanitary sewer infrastructure so it is possible there are sewer pipe leaks into streams. Petroleum-based hydrocarbons from urban landscapes may also be responsible for inputs of small, labile fractions of DOM from urbanized or suburbanized landscapes (McElmurry et al., 2013).

*Microbial Use and DOM Quality.* Repeated-measures ANOVA comparing bioavailability between urbanized and forested sites incorporating seasons (spring/summer, n=2; fall/winter, n=3) revealed an interaction between season and watershed land cover ( $F_{(1,6)}=15.7$ ,  $p<0.01$ ). Bioavailability was significantly higher in urbanized versus forested streams but only during sampling events in spring and summer (Tukey's HSD,  $p<0.05$ ; Figure 1.6).

To determine if seasonal variation in DOM bioavailability was associated with DOM composition, these variables were compared by season using repeated-measures ANCOVA. Protein-like components, C4 and C5, were combined as a single variable, "protein-like DOM," for bioavailability analysis following a previous approach (Fellman et al., 2008). Significant seasonal interactions were found when bioavailability was compared to percent fluorescence of PARAFAC components C1 and C2, percent protein-like components, HIX, and  $SUVA_{254}$  (Table 1.4).

Surprisingly, significant relationships between carbon quality and percent BDOC were only found for samples collected in warmer months with no significant relationships identified in colder seasons. Reflecting lower bioavailability of humic substances, PARAFAC components C1 and C2 as well as HIX and  $SUVA_{254}$  were negatively correlated with bioavailability for samples

collected in spring and summer (Figure 1.7 a, b, f, and h). Percent fluorescence of protein-like DOM was positively correlated to percent BDOC in the spring and summer (Figure 1.7 d), as expected for this typically labile material.

These results only partially support the second hypothesis that DOM from urban watersheds would be more readily used by microbes because higher bioavailability was only seen during warmer seasons. During colder months microbial use was significantly lower and seemingly decoupled from DOM quality. This suggests that other factors such as nutrient limitation or changes in the heterotrophic activity of the microbial inoculum were responsible for the seasonal differences in DOM bioavailability.

To test for a seasonal nutrient limitation effect, repeated-measures ANCOVA analysis was used to compare BDOC to each fluorescence metric used in this study with dissolved N:P ratios included as a covariate. Sample N:P ratio did not approach significance as a covariate for any of PARAFAC component. Stream water N:P ratios were significantly greater in urbanized streams compared to forested streams across all seasons ( $p < 0.001$ ) and the urban N:P ratios did not differ between seasons (Figure 1.6).

*Microbial Use of DOM and Heterotrophic Activity.* For each bioassay sampling date, a fresh microbial inoculum was collected. Microbial community composition in streams can vary seasonally (Hullar et al., 2006); thus, if the heterotrophic activity of microbial inocula changed between sampling dates, this may explain observed differences in bioavailability predicted by Hypothesis 3. To test this hypothesis, samples for EEA

analysis were taken from site P1 contemporaneously with microbial inoculum collection. Activity of all three enzymes measured – aminopeptidase, phosphatase, and esterase – was greatest in April and August 2012 (Figure 1.8a). Increasing EEA coincided with increased percent BDOC in both urbanized and forested stream water samples (Figure 1.8 b-d). These results are limited but suggest that seasonal changes in microbial activity can be an important factor determining microbial use of DOM.

*Ecosystem Implications.* Increasing watershed impervious cover was related to a shift from complex, recalcitrant DOM to smaller, more microbially available compounds. These observed changes are related to increased production of microbially sourced DOM in urbanized streams and watersheds that lack point source inputs. This work supports my first two hypotheses and bolsters suggestions that non-point source effects of urbanization are altering organic carbon dynamics both across watersheds and in stream ecosystems (Imberger et al., 2014; McElmurry et al., 2013; Kaushal et al., 2014).

Similar relationships between DOM composition, as measured by fluorescence spectroscopy and bioavailability, have been reported elsewhere (Fellman et al., 2008; Cory and Kaplan, 2012; Petrone et al., 2011); but see (Lu et al., 2013b) for contrasting results); however to my knowledge this study is the first to link such changes to urbanization via impervious cover exclusively. The results presented here also demonstrate higher BDOC in stream water samples during summer months and decreased bioavailability in winter months in streams impacted by urbanization. This seasonal variation has implications for headwater and downstream systems alike. Such

increased variability and altered timing of DOM export may interact with other seasonal dynamics in unpredictable ways and represents an important avenue for future research.

Direct evidence of ecosystem impacts in urbanized streams due to altered carbon composition is limited. Experimental additions of labile DOM sources to forested streams demonstrated increases in bacterial production and respiration (Bernhardt and Likens, 2002) and subsequent transfer of organic carbon to higher trophic levels (Hall and Meyer, 1998). More research is required to determine if urban stream processes respond similarly to increased lability of organic carbon. The change observed in DOM composition and bioavailability in urbanized streams of the Parkers Creek watershed has significant implications for understanding functioning of many stream ecosystems, particularly because these urbanized watersheds lack the point source and septic inputs that were assumed to be major sources of anthropogenic DOM in streams. The fact that increased presence of altered DOM found in urbanized streams was related to increased heterotrophic use of DOM during spring and summer months also has substantial implications because increases of stream DOM lability have been shown to change stream nutrient dynamics (Bernhardt and Likens, 2002), re-direct organic carbon to bacterial growth and respiration (Bernhardt and Likens, 2002), and alter the taxonomic composition of the heterotrophic microbial communities that rely on this energy source (Judd et al., 2006; Kirchman et al., 2004). Ultimately, the fate of stream organic carbon changes significantly, from being transported downstream to being diverted directly to microbial production and respiration (Battin et al., 2008), potentially resulting in greater CO<sub>2</sub> exports from urbanized streams. As urban populations globally continue to expand

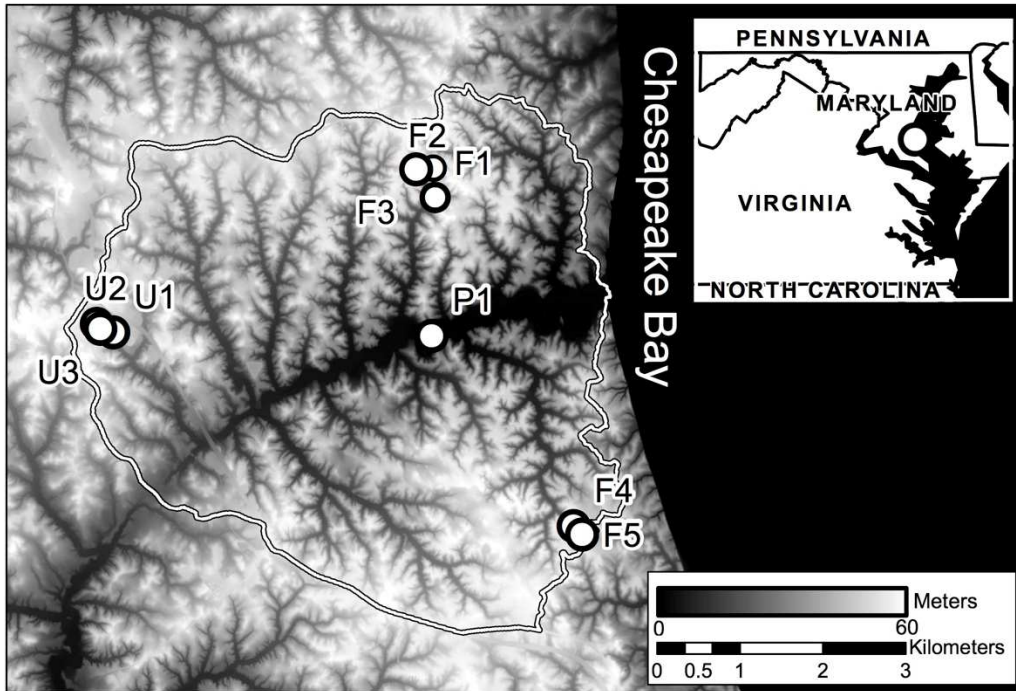
(World urbanization prospects: the 2011 revision, 2012), increased stream DOM bioavailability may impact not only individual streams but the carbon cycle at large.

## Tables and Figures

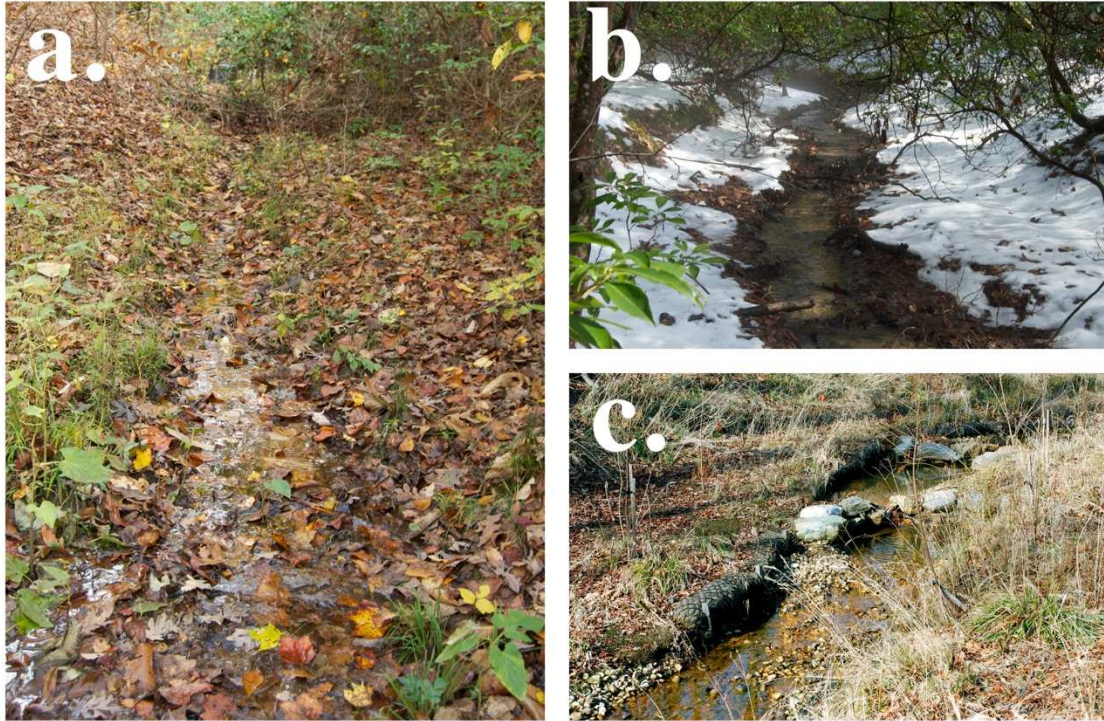
**Table 1.1:** Study site locations, designations, and landscape statistics.

Site	Site Designation	Latitude	Longitude	% Impervious Cover	% Forest Cover	% Agriculture Cover	Total Hectares	# Septic Systems
F1	Forested	38°32'50.63"N	76°32'29.48"W	0.40%	90.6%	0.00%	11.7	0
F2	Forested	38°33'2.08"N	76°32'30.28"W	4.95%	64.4%	0.00%	4.18	0
F3	Forested	38°33'1.68"N	76°32'38.71"W	2.06%	94.7%	3.65%	6.97	0
F4	Forested	38°30'41.86"N	76°31'21.18"W	0.00%	100%	0.00%	2.86	0
F5	Forested	38°30'38.50"N	76°31'17.04"W	2.02%	90.8%	6.03%	2.78	0
U1	Urbanized	38°31'58.49"N	76°35'10.01"W	44.0%	33.5%	0.00%	1.53	0
U2	Urbanized	38°32'1.75"N	76°35'18.21"W	24.4%	27.7%	0.00%	7.71	2
U3	Urbanized	38°32'1.17"N	76°35'17.60"W	10.0%	2.50%	0.00%	4.69	1
P1	Main Stem	38°31'56.56"N	76°32'31.41"W	7.42%	71.4%	7.86%	2181.7	288





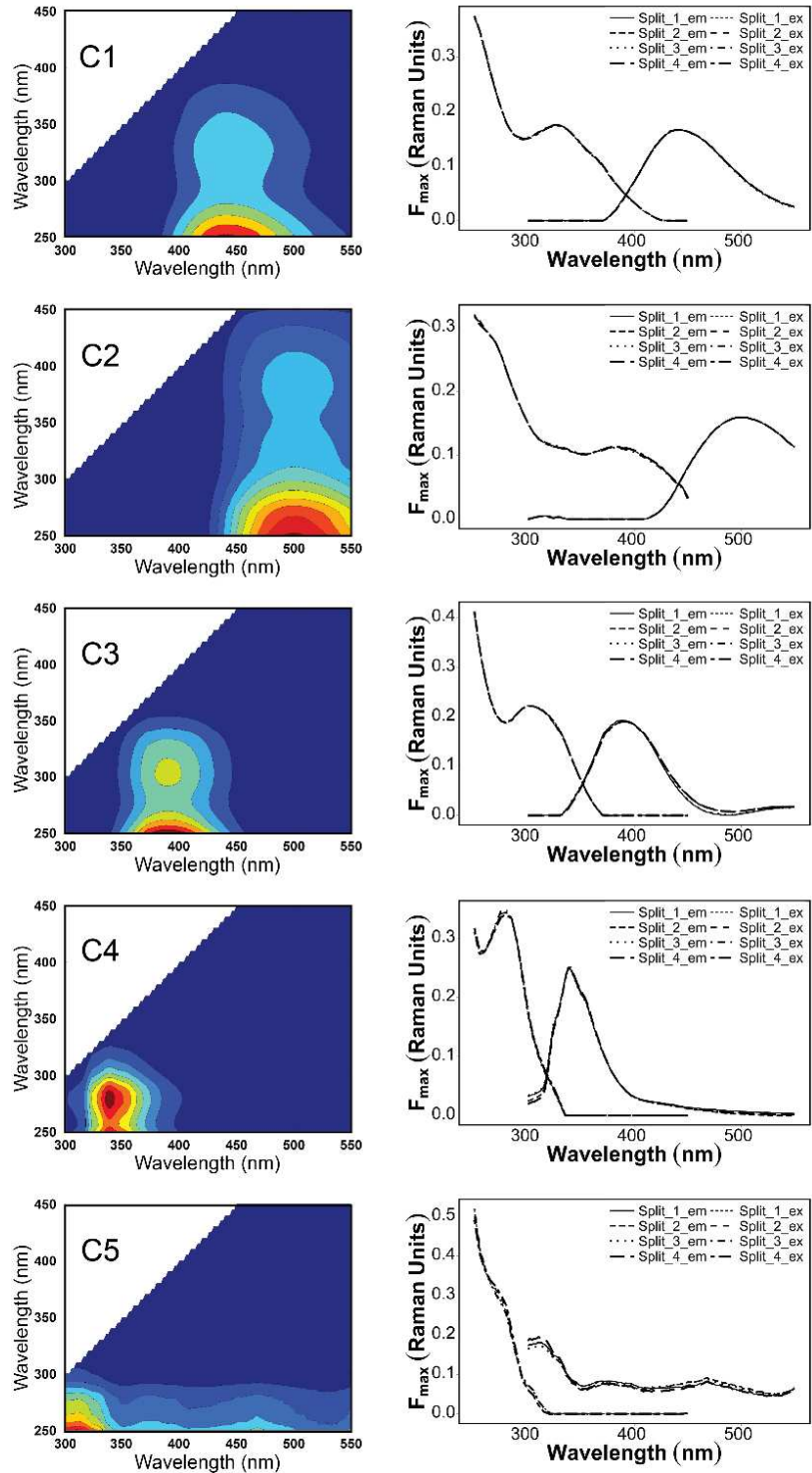
**Figure 1.1:** A map based on a digital elevation model (DEM) of the Parkers Creek watershed with study locations indicated. Inset: map of Maryland and surrounding states with the location of the study watershed indicated. Source: LiDAR data provided by Calvert County Government. Inset sources: Esri, TomTom, U.S. Department of Commerce, U.S. Census Bureau.



**Figure 1.2:** Representative images of stream sites. Photographs are of sites (a) F4, (b) F2, and (c) U2.

**Figure 1.3:**

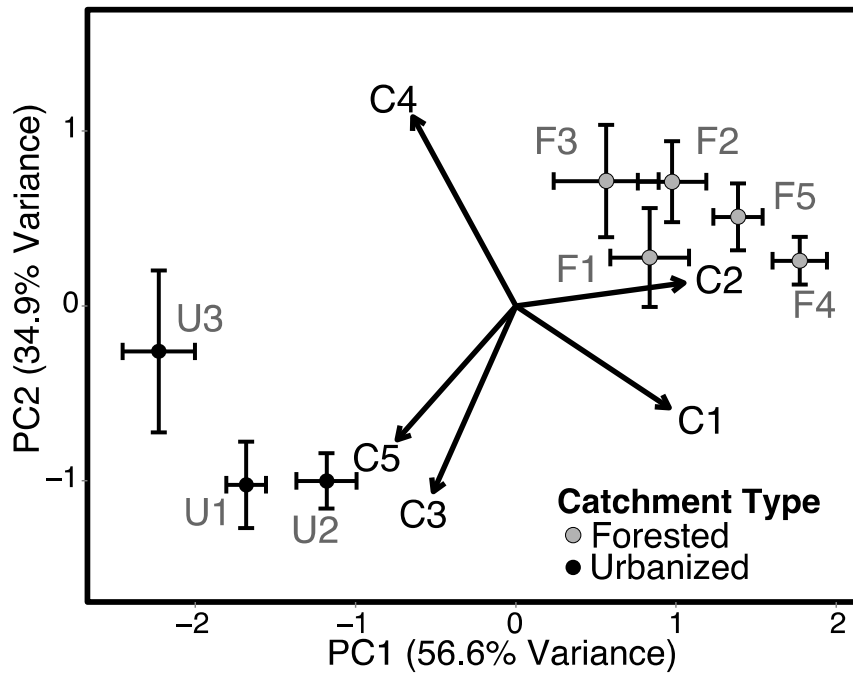
Excitation-emission plots (left column) of the five EEM-PARAFAC components (C1, C2, C3, C4, C5) validated in this study. Red and blue hues indicate higher and lower fluorescence intensities, respectively. Line plots (right column) represent the results of the four split-half validation models that were produced for each of the five components.



**Table 1.2:** The emission and excitation maxima and characteristics of the modeled EEM-PARAFAC components.

<b>Component</b>	<b>Excitation Maxima (nm)</b>	<b>Emission Maxima (nm)</b>	<b>Description</b>
C1	<250, (325)	438	Humic-like fluorophore of terrestrial origin (Stedmon et al., 2003; Stedmon and Markager, 2005), highest concentrations in forest streams, wetlands, and agriculturally influenced streams (Osburn et al., 2012), absent from wastewater (Stedmon and Markager, 2005), photolabile.
C2	<250, (380)	502	Ubiquitous fulvic acid (Stedmon and Markager, 2005), Recalcitrant, aromatic, terrestrial humic (Lutz et al., 2012), humic acid-like UVC/UVA excitation (Stedmon et al., 2003; Osburn et al., 2012), possibly indicative of biogeochemical processing of terrestrial particulate organic matter (Yamashita et al., 2008).
C3	<250, (300)	388	An anthropogenic humic fluorophore dominant in wastewater DOM and linked to DOM originating in agricultural catchments (Stedmon and Markager, 2005), likely of microbial origin (Yamashita et al., 2008; Cory and McKnight, 2005), similar to marine humic-like fluorophores (Yamashita et al., 2008; Coble, 1996).
C4	<250, 280	340	Tryptophan-like, protein-like fluorescence (Stedmon et al., 2003; Osburn et al., 2012; Coble, 1996) indicative of recent production (Osburn et al., 2012).
C5	<250	310	Tyrosine-like, protein-like fluorescence (Fellman et al., 2008; Coble, 1996).

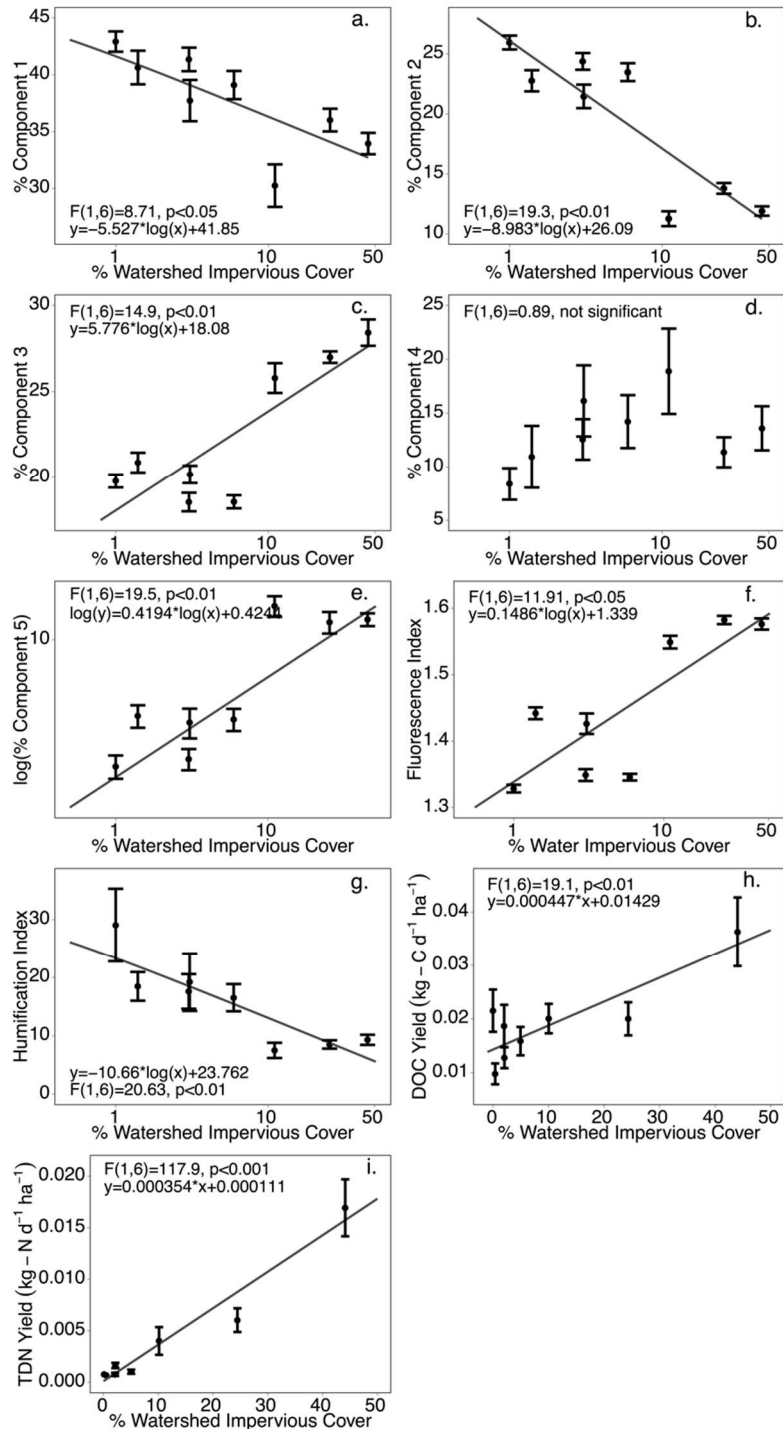
Previous studies that have described EEM-PARAFAC components with similar properties were identified. Based on this review, a description of the likely characteristics of each DOM fluorophore identified was developed.



**Figure 1.4:** Principal component analysis (PCA) of DOM composition of forested (F1, F2, F3, F4, F5) and urbanized (U1, U2, and U3) stream sites as measured by percent Fmax of DOM PARAFAC components (C1, C2, C3, C4, and C5). Error bars indicate standard error at each site for principal components 1 and 2. The percentages indicated in the axis titles indicate the percent variance explained by each principal component of the PCA.



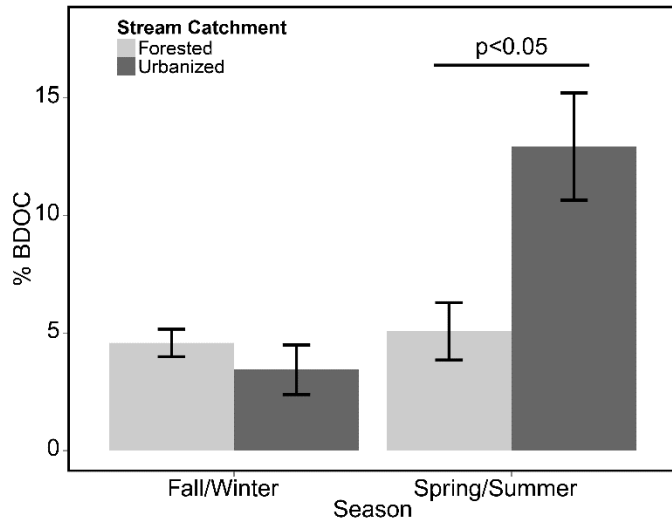
**Figure 1.5:** Percent catchment impervious cover as a measure of non-point source urban impacts versus mean value by stream sampling site of: (a) percent Fmax Component 1, (b) percent Fmax Component 2, (c) percent Fmax Component 3., (d) log-transformed percent Fmax Component 5, (e) Fluorescence Index, (f) Humification Index, (g) watershed DOC yield, (h) watershed TDN yield, and (i) SUVA<sub>254</sub>. Impervious cover was related to water chemistry using repeated-measures



linear regression. Error bars represent standard error of the mean by site.

**Table 1.3:** Results for the repeated measures linear regression analysis of stream water chemistry variables compared to catchment impervious cover.

<b>Dependent Variable</b>	<b>Independent Variable</b>	<b>num. df</b>	<b>den. df</b>	<b>F-value</b>	<b>p-value</b>	<b>slope</b>	<b>intercept</b>
% C1	log(% impervious cover)	1	6	8.71	0.0255	-5.527	41.85
% C2	log(% impervious cover)	1	6	19.3	0.0046	-8.983	26.09
% C3	log(% impervious cover)	1	6	14.9	0.0084	5.776	18.08
% C4	log(% impervious cover)	1	6	0.89	0.3817		
log(% C5)	log(% impervious cover)	1	6	19.5	0.0045	0.4194	0.4244
FI	log(% impervious cover)	1	6	11.9	0.0136	0.1486	1.339
HIX	log(% impervious cover)	1	6	20.6	0.0039	-10.66	23.76
DOC Conc.	log(% impervious cover)	1	6	4.44	0.0797	-0.8701	2.886
TDN Conc.	log(% impervious cover)	1	6	12.8	0.0117	0.3507	0.02123
DOC flux	log(% impervious cover)	1	6	0.22	0.6581		
DOC yield	% impervious cover	1	6	19.1	0.0047	0.000447	0.01429
TDN flux	% impervious cover	1	6	9.99	0.2383		
TDN yield	% impervious cover	1	6	118	<0.0001	0.000354	0.000111



**Figure 1.6:** Comparison of the percent DOM lost over 28 days by season ("Fall/Winter" and "Spring/Summer") and between streams in urbanized and forested catchments. Comparisons were made using Tukey's HSD. Error bars represent standard error of the mean.



**Figure 1.7:** Percent

BDOC compared to  
DOM spectroscopy and  
water chemistry

variables from individual  
water samples. Statistical  
comparisons were made

between BDOC and (a)  
percent PARAFAC

component C1, (b)

percent PARAFAC

component C2, (c)

percent PARAFAC

component C3, (d)

percent fluorescence of

protein-like PARAFAC

components, (e) the

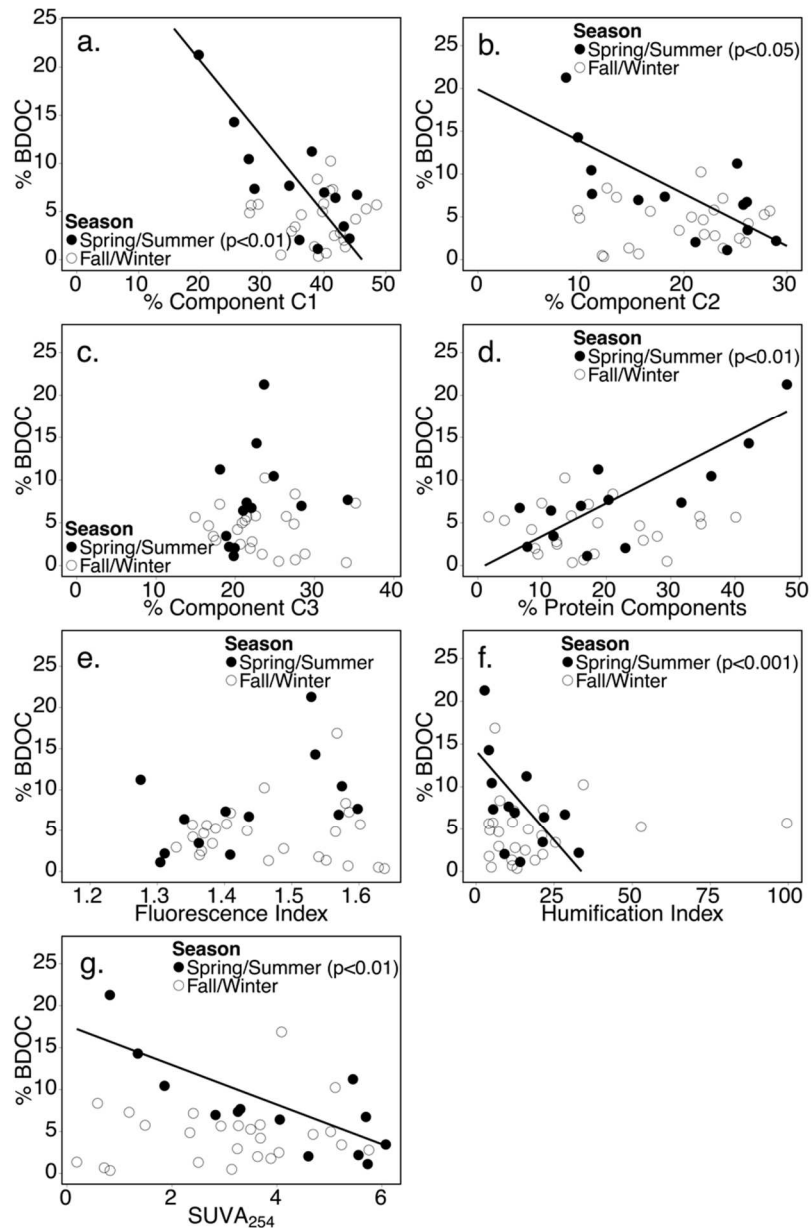
fluorescence index, (f) the humification index, and (g) SUVA<sub>254</sub>. Statistics presented are

repeated-measures ANCOVA tests. A significant interaction across season was found

when percent BDOC was compared to percent PARAFAC component C1, percent

PARAFAC component C2, percent protein-like components, humification index values,

and SUVA<sub>254</sub>.

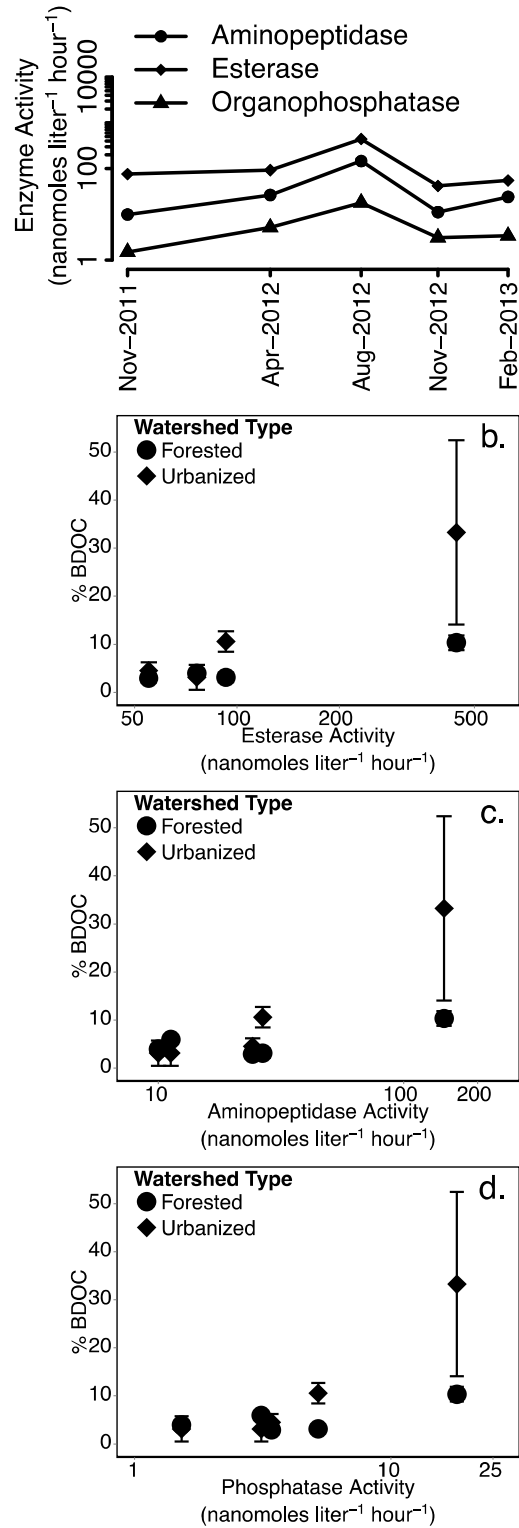


**Table 1.4:** Statistical comparisons of DOM spectroscopy metrics to percent DOM loss using repeated measures ANCOVA.

Dependent Variable	Independent Variable 1	Independent Variable 2	Interaction num. df	Interaction den. Df	Interaction F-value	Interaction p-value
% BDOC	Percent Component C1	Season	1	27	14.4	0.0008
% BDOC	Percent Component C2	Season	1	27	14.3	0.0008
% BDOC	Percent Component C3	Season	1	27	2.51	0.1248
% BDOC	Percent Protein-Like DOM	Season	1	27	23.3	<0.0001
% BDOC	Fluorescence Index	Season	1	27	8.40	0.0075
% BDOC	Humification Index	Season	1	27	13.0	0.0012

Statistics reported below are the interaction between Independent Variable 1 and Independent Variable 2.

**Figure 1.8:** On each bioassay sampling date, an inoculum collected at a common downstream site was amended to the samples at the start of incubations (i.e., a common inoculum was used across sites but varied by date). Aminopeptidase, organophosphatase, and esterase enzyme activities of water collected from site P1 are (a) plotted over time. Activity of (b) phosphatase, (c) esterase, and (d) aminopeptidase extracellular enzymes associated with the microbial inoculum were compared to percent BDOC for all incubations conducted on that date. BDOC of urbanized and forested streams were considered separately. Error bars represent percent BDOC standard error across streams of a given watershed type.



## Chapter 2: Stream water temperature and groundwater chemistry impact microbial extracellular enzyme activity in headwater streams.

### **Introduction**

Organic matter has long been recognized as a central food resource for aquatic food webs (Lindeman, 1942). Entry of dissolved and particulate organic matter into stream food webs occurs via heterotrophic microbial uptake and assimilation of these resources. Once assimilated, organic matter can be passed to higher trophic levels in streams (Bernhardt and Likens, 2002), and ultimately terrestrial environments (Baxter et al., 2005).

Heterotrophic microbial communities responsible for carbon uptake are diverse and known to deploy an array of metabolic strategies to access organic matter for mineralization and biological assimilation. In fact, microbes are responsible for the largest proportion of metabolic activity in streams and rivers (Logue et al., 2004; Marxsen, 2006).

Most of the organic matter compounds utilized by stream microbes are macromolecule polymers, most commonly humic substances; ranging in size between 500 and 10,000 kiloDaltons (Thurman et al., 1982). Since these compounds are typically too large to be transferred directly across microbial cell membranes, microbes produce extracellular enzymes to break down these molecules for uptake (Chróst, 1991). Microbial metabolism of the organic macromolecules is limited by extracellular enzyme processing rates, rather than the uptake process itself (Hoppe, 1983; Chróst and Rai, 1993) meaning that measurements of extracellular enzymes are meaningful measures of microbial

heterotrophic activity. To measure extracellular enzyme activity (EEA), stream water samples are incubated with fluorescently labeled substrates. As substrates are hydrolyzed, fluorophores are released, providing a measure of enzyme activity levels.

Reflective of the rate-limiting nature of extracellular enzymes, EEA measures have been linked to a number of microbial functions including respiration (Sinsabaugh and Findlay, 1995; Margesin et al., 2000; Sinsabaugh and Shah, 2010), nutrient uptake (Hill et al., 2010b), and organic matter mineralization in both aquatic and terrestrial ecosystems (Sinsabaugh, 1994; Alvarez and Guerrero, 2000; Lehto and Hill, 2013). For this reason, EEA can be applied as a representative measure of in-stream microbial metabolic activity and identifying what controls EEA is a very active area of research. This research is critical to advancing understanding of the role microbial heterotrophic activity plays in freshwater biogeochemical cycles and more generally the fate of organic carbon in fluvial systems (Battin et al., 2008; Wilson and Xenopoulos, 2008).

At the cellular level, the induction of an extracellular enzyme occurs through a complex set of interacting factors that respond to both the presence of a resource as well as microbial demand for that resource (Sinsabaugh and Moorhead, 1994; Hill et al., 2010b; Sinsabaugh and Follstad Shah, 2012). Studies of microbial EEA in freshwater environments have found a relationship between extracellular enzyme activities and a variety of environmental variables including organic matter supply (Findlay et al., 1997), pH (Chen et al., 2012), conductivity (Neubauer et al., 2013), and temperature (Koch et al., 2007; Sinsabaugh and Shah, 2010; Sinsabaugh and Follstad Shah, 2012). Study

results; however, have been quite variable and in some cases contradictory (Arnosti et al., 2014). Nutrient resource stoichiometry appears to be a primary factor driving EEA (Hill et al., 2010b, 2012; Lang et al., 2012; Sinsabaugh et al., 2012; Sinsabaugh and Follstad Shah, 2012), yet there remains substantial variation in enzyme activities that stoichiometry does not explain.

Enzyme kinetics are also tightly linked to temperature (Chróst, 1991). The result is that temperature modulates the response of microbial communities to environmental conditions (Steen and Arnosti, 2014; Ylla et al., 2014). Fortunately, models exist that can incorporate the interacting effects of temperature and environmental complexity on enzymatic processing rates. Control of temperature on environmental extracellular enzymes has been described via application of metabolic scaling theory (Gillooly et al., 2001; Brown et al., 2004) in combination with Michaelis-Menten enzyme kinetics (Sinsabaugh and Shah, 2010; Sinsabaugh and Follstad Shah, 2012). These principles can be applied to calculate the apparent activation energy ( $E_a$ ) of suites of enzymes in varying environments. In the context of analysis of environmental samples, where the aggregate activity of many enzymes are being measured at once under substrate saturating conditions,  $E_a$  measurements are largely indicative of temperature dependence of enzyme activity (Davidson and Janssens, 2006; Sinsabaugh and Shah, 2010; Sinsabaugh and Follstad Shah, 2012). Enzyme temperature dependence data from streams are very limited, but experimental evidence found that enzyme processing rates of some enzymes increased more quickly with temperature than would be predicted by enzyme kinetics alone, indicating an interactive role between enzyme activity and environment (Ylla et

al., 2014). Thus, there is a need to unravel the interactive effects of stream environmental conditions and temperature on microbial metabolic processes (Sand-Jensen et al., 2007).

Landscape urbanization has been related to extracellular enzyme activity; with results that conflict across studies. Some research has shown a positive relationship between enzyme activity and urbanization (Harbott and Grace, 2005; Williams et al., 2011) while others have shown a negative relationship to anthropogenic impacts (Hill et al., 2012; Lehto and Hill, 2013). Urbanized streams typically have elevated temperatures and water conductivity as well as altered organic carbon quality (Walsh et al., 2005; McElmurry et al., 2013; Imberger et al., 2014; Hosen et al., 2014), all of which have been linked to changes in stream microbial EEA. Different results between studies may be also driven by the relative dominance of competing stimulatory (e.g. increased nutrient loads) and inhibitory (e.g. chloride) factors at a streams site.

My goal was to measure EEA in streams along a gradient of urbanization across multiple years to determine if enzyme activities varied: 1) between urbanized and forested headwater streams and 2) across seasons at stream sites.

I tracked seven enzyme activities as well as a suite of environmental parameters that included watershed land cover and stream water chemistry across a group of first-order sub-watershed within a single larger watershed in the Coastal Plain of Maryland, USA. This was meant to eliminate interregional differences among sub-watersheds due to climate and physiographic province.

Prior work on Parkers Creek urbanized streams has shown that nutrient N:P ratios and bioavailable dissolved organic matter levels exceed those of forested streams (see Chapter 1). Based on this evidence, I anticipated that inhibitory effects of urbanization would be outweighed by resource stimulation. Overall, I hypothesized that EEA would be greater in urbanized than forested streams, particularly for enzymes that access carbohydrate resources (e.g. alpha- and beta-glucosidase) and organic phosphorus (e.g. alkaline phosphatase).

Regarding seasonal trends, I hypothesized that streams would exhibit predictable seasonal trends in EEA across both forested and urbanized sites with higher EEA levels in spring and summer months in response to increasing temperatures. It was also predicted that carbohydrate-seeking enzyme levels would be highest in November in response to inputs of bioavailable carbon from leaf fall.

## **Methods**

*Study sites.* The Parkers Creek watershed is located on the Western Shore of the Chesapeake Bay in the Coastal Plain of Maryland, USA, where streams are composed of alluvial materials and represent a gradient of land use. A total of eight first-order streams were studied (Figure 2.1, Table 2.1). Five of these were forested (F1, F2, F3, F4, F5) and three were urbanized (U1, U2, and U3). Samples were collected at all sites on May 2011, July 2011, November 2011, February 2012, April 2012, August 2012, November 2012, and February 2013, with the exception of August 2012 when sites F1 and F4 were dry.



*Physicochemical variables.* Streamwater temperature, specific conductivity, pH, and dissolved oxygen were measured in the field using a YSI Professional Plus multiparameter meter (YSI Inc.; Yellow Springs, OH). Samples for dissolved organic carbon (DOC) concentration and DOM quality measurements were filtered in the field with 0.7 µm GF/F filters (Whatman Inc., Maidenstone, UK) and were stored in borosilicate amber glass bottles with Teflon-coated lids. Sample bottles were acid washed in 10% HCl and both bottles and GF/F filters were combusted for 4 hours at 450°C. Water for other analysis was filtered in the field into amber HDPE plastic bottles. All samples were returned to the laboratory on ice. Samples for carbon quality were kept at 4 °C and were analyzed within 72 hours of collection. Other samples were frozen until processing at a later date.

Total DOC, as non-purgeable organic carbon, and total dissolved nitrogen (TDN) were determined by analysis on a Shimadzu TOC-vCPH with attached TNM-1 unit (Shimadzu Corporation; Kyoto, Japan). Carbon quality was determined using the fluorescence index (FI), which is an indicator that DOM in a water sample is primarily allochthonous or autochthonous (McKnight et al., 2001). Fluorescence data were collected on a Horiba Scientific Fluoromax-4 as described previously (see Chapter 1). The fluorescence index was determined as the ratio of fluorescence emission intensities at 450 and 500 nm when a water samples was excited at 370 nm (McKnight et al., 2001).

Dissolved N:P ratios were calculated using the ratio of dissolved inorganic nitrogen to dissolved orthophosphate-phosphorus (DIN:DOP). Dissolved inorganic nitrogen was determined as the sum of dissolved ammonium and nitrate. A Lachat QuikChem 8500 Series 2 flow injection analyzer was used to determine dissolved nitrate, ammonium, and orthophosphate concentrations. Total dissolved sulfate and chloride concentrations were determined using a Dionex ICS-1000 ion chromatograph.

Samples for total iron and cation analysis were filtered to 0.2  $\mu\text{m}$  and acidified to pH 2 using nitric acid. Total iron was determined following Hach FerroVer kits (Hach Company; Loveland, CO). Water samples were analyzed for Na, Ca, K, Mg, and Sr using a PerkinElmer 8300 inductively coupled plasma-optical emission spectrometer (PerkinElmer, Inc.; Waltham, MA).

*Extracellular enzyme activity (EEA).* Estimates of EEA were determined by measuring the rate of degradation of a suite of model organic substrates by enzymes present in whole water samples. Work was conducted based on standard methods (Sinsabaugh and Findlay, 1995; Findlay et al., 2001; Findlay, Stuart, 2007) as reported in Chapter 1.

Whole water samples for EEA analysis were collected in acid-washed polycarbonate bottles from the field sites described above. Samples were returned to the laboratory on ice and were stored in the laboratory at 4 °C. Extracellular enzyme activity analysis was conducted within 24 hours of collection.

A total of seven model substrates, labeled with fluorogenic substrates, representing a range of organic carbon substrates commonly used by microbial communities, were supplied to stream water samples and their microbial communities (Table 2.2). Substrates were prepared in autoclaved 5 mM bicarbonate buffer with the exception of 4-MUB-acetate and L-Leucine-7-amido-4-methylcoumarin hydrochloride, which were prepared in autoclaved nanopure water. To ensure complete dissolution, substrates were stirred in the dark at 4 °C for 24 hours before the experiment. Experimental incubations were conducted in black 96-well plates. Substrate and stream water were added in equal amounts (150 µL each) to wells with a final substrate concentration of 500 µM. Each substrate/sample combination was replicated four times. Each 96-well plate included replicated controls of streamwater, buffer or nanopure water, and labeled substrate. A standard curve based on coumarin was developed for the incubations using L-Leucine7-amido-4-methylcoumarin hydrochloride. For all other enzymes, a standard curve using 4-methylumbelliferone was applied. Standard curves were replicated four times on each analysis date. Microplates were incubated in the dark for a total of 4 hours at 20 °C. Fluorescence was measured at regular intervals at an excitation wavelength of 365 nm and emission wavelength of 450 nm using a Molecular Devices SpectraMAX Gemini XPS plate-reading spectrofluorometer. Following each experimental incubation, sample photoquenching was assessed by spiking buffer and streamwater controls with 50 µL of the relevant 1 µM standard. All labeled substrates and standards were purchased from Sigma-Aldrich.

*Landscape Data.* Landscape characteristics were obtained from light detection and ranging (LIDAR) data provided by Calvert County, Maryland government. A digital elevation model (DEM) was developed from the LIDAR data. The resulting DEM was used to delineate watersheds and determine watershed areas of associated with each stream sampling site. All landscape analysis was conducted in ArcGIS 10.1. Watershed land cover analysis was conducted manually using digital orthophotography provided by Calvert County, Maryland government. Stream maps were obtained from the National Hydrography Dataset (NHD) Plus Version 2.10.

*Data Analysis.* For all statistical analysis an alpha of 0.05 was adopted *a priori*. Using R 3.1.1 (Oksanen et al., 2013), principal component analysis (PCA) was conducted using the *princomp* function and canonical correlation analysis (CCA) conducted using the *Vegan* package. Statistical significance of canonical correlations was calculated using Bartlett's chi-squared. Longitudinal replicates were excluded from PCA and CCA analysis to avoid potentially interactive effects related to watershed scale. Repeated-measures analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were determined using the *Proc Mixed* function in SAS 9.3. When multiple independent factors were considered, interaction terms were always included. If an interaction term was not significant, it was removed from the final model. Normality was assessed and those variables that deviated substantially from normality log-transformed; all enzyme activities had to be log-transformed. Because there was a significant seasonal interaction in the relationship between temperature and EEA for four enzymes studied ( $\alpha$ Gase,  $\beta$ Gase, NAGase, XYLase), piecewise regression analysis (using the *Segmented* package

in R 3.1.1) was applied to determine the breakpoint at which the relationship between temperature and EEA changed (Muggeo, 2003).

For enzyme activities that were significantly correlated with stream water temperature at time of collection, temperature sensitivity was evaluated using the  $Q_{10}$  coefficient, describing how this activity changes with an increase of 10° C (Lloyd and Taylor, 1994):

$$B = \log_{10} Q_{10}/10 \quad (1)$$

Where B is the slope of the regression equation for temperature and the relevant EEA. After computing the  $Q_{10}$  values, I estimated activation energies using the following equation, adapted from Gillooly et al. (2001) using a modified version of the Van't Hoff-Arrhenius equation (Arrhenius 1915):

$$Q_{10} = e^{[E/(kT_0^2)] \times 10} \quad (2)$$

$$E = \frac{\ln Q_{10}}{10} \times kT_0^2 \quad (3)$$

The equations rely on absolute values of temperature (T, degrees Kelvin) along with the Boltzmann constant ( $k$ , eV) and previously computed  $Q_{10}$  values to estimate apparent activation energy ( $E$ , eV K<sup>-1</sup>).

## Results

*Environmental Controls on EEA.* Canonical correlation analysis was conducted to link enzyme activities to independent environmental factors. Log-transformed activity of all seven enzymes assessed was compared to a suite of physicochemical factors (Figure 2.1). Canonical correlation analysis was conducted on data from eight first order study sites (F1, F2, F3, F4, F5, U1, U2, and U3). A complete summary of EEA levels for the sites is included in Appendix 1.

Previous analysis from a Parkers Creek main stem site showed that enzyme activity profiles could best be separated into two groups, cold seasons and warm seasons (see Chapter 1). Based on this evidence, CCA was conducted on two subsets of the data, one that included fall and winter samples (November 2011, February 2012, November 2012, and February 2013) and another that included spring and summer samples (May 2011, July 2011, April 2012, August 2012,). The results of this analysis showed that for both cold (Fall/Winter) and warm (Spring/Summer) season samples, temperature was the environmental factor most strongly linked to overall enzyme activity (Figure 2.2). Interestingly, while temperature was positively related to most enzyme activities in the Fall/Winter, the opposite was true in Spring/Summer with the relationship being largely negative, indicating optimal processing at moderate temperatures.

*Temperature Controls on Metabolic Activity.* The apparent seasonal difference in the relationship between temperature and enzyme activity was tested directly by repeated-measures ANCOVA. Log-transformed enzyme activity, the independent variable, was

compared to temperature with season (“Fall/Winter” or “Spring/Summer”), which was included as a covariate. Temperature was significantly related to enzyme activity for six out of the seven enzyme substrates studied. The activities of two enzymes, esterase ( $F_{(1,52)}=8.68$ ;  $p<0.01$ ) and phosphatase ( $F_{(1,52)}=7.7$ ;  $p<0.01$ ), were positively related to temperature across all seasons (Figure 2.3c and e). For the remaining four enzyme types, a seasonal interaction was observed. In fall and winter,  $\alpha$ Gase ( $F_{(1,23)}=26.5$ ,  $p<0.001$ ; Figure 2.3a) and NAGase ( $F_{(1,23)}=26.1$ ,  $p<0.001$ ; Figure 2.3d) activities were positively related to temperature, but demonstrated no significant relationship in warm season samples. For the remaining two enzymes,  $\beta$ Gase (Figure 2.3b) and XYLase (Figure 2.3f), there was a significant positive relationship between temperature and enzyme activity in fall and winter samples ( $\beta$ Gase:  $F_{(1,23)}=13.0$ ,  $p<0.01$ ; XYLase:  $F_{(1,23)}=10.36$ ,  $p<0.01$ ) and a negative relationship in spring and summer samples ( $\beta$ Gase:  $F_{(1,20)}=8.52$ ,  $p<0.01$ ; XYLase:  $F_{(1,20)}=7.47$ ,  $p<0.05$ ). For those enzymes with a significant seasonal interaction, piecewise regression was performed to determine the breakpoint at which the relationship between enzyme activity and temperature changed. Significant breakpoints were found for all four enzymes assessed –  $\alpha$ Gase,  $\beta$ Gase, NAGase, and XYLase – ranging from 11.2 to 14.4 °C (Table 2.4).

To test the temperature sensitivity of microbial metabolism,  $Q_{10}$  and apparent  $E_a$  values were computed for each group of enzyme assayed (Table 2.5). Values of the  $Q_{10}$  temperature sensitivity metric indicate the factor by which a functional process, in this case enzyme activity, changes with a temperature change of 10 °C (Koch et al., 2007; Sinsabaugh and Follstad Shah, 2012). This analysis indicated that the enzymes assayed

fell into two groups;  $\alpha$ Gluc,  $\beta$ Gluc, and XYLase were all highly temperature sensitive ( $Q_{10}=4.19-5.57$ ) while esterase, NAGase, and APase were much less responsive to temperature changes ( $Q_{10}=1.43-2.57$ ).

*Temperature Drives Seasonal Changes in EEA.* Given the large impact of temperature on EEA as measured by CCA, I assessed how temperature influenced seasonal trends in enzyme activity, repeated-measures ANOVA was conducted on log-transformed enzyme activities with season (“Spring”, “Summer”, “Fall”, or “Winter”) and watershed type (“Forested” or “Urbanized”) as independent variables. Each of the six enzymes that were significantly correlated to temperature also displayed significant changes across sampling seasons (Figure 2.4). Enzyme activities were generally lower in winter than other seasons. Activities were significantly lower in winter than at least one other season for  $\alpha$ Gase, esterase, NAGase, phosphatase, and XYLase enzymes (Figure 2.4a, c, d, e, and f). Lower activity in winter reflects the significant negative relationship between temperature and enzyme activity during cold weather months.

There was also a noticeable drop in activity rates of some enzymes during the hottest season, summer. Activity was significantly lower in summer than spring and fall for XYLase enzymes (Figure 2.4f) with a substantial but non-significant decrease in  $\alpha$ Gase activities during summer compared to other seasons. The activities of  $\beta$ Base and NAGase were also significantly depressed in urbanized streams on summer sampling dates (Figure 2.4b and d). This decreased enzyme activity during the summer in urbanized sites may be explained by the significantly higher temperatures found across all seasons for



urbanized versus forested sampling sites ( $F_{(1,6)}=8.45$ ;  $p<0.05$ ). The four enzymes that showed evidence of reduced activity during summer months ( $\alpha$ Gase,  $\beta$ Gase, NAGase, and XYLase) were the four enzymes for which a seasonal interaction was found for the relationship between activity and temperature. By contrast, the activity of the two enzymes that were positively related to temperature across all sampling seasons, esterase and phosphatase, did not show any decrease in enzyme activity during summer sampling campaigns.

*Land Use and Enzyme Activity.* Phosphatase was the only enzyme for which EEA values were significantly higher in urbanized streams than forested streams across all seasons ( $F_{(1,6)}=28.93$ ,  $p<0.01$ ). The CCA provided insight into the underlying factors responsible for increased phosphatase activity in urbanized streams. Stream water conductivity and carbon quality, as measured by the fluorescence index, were important factors in both Fall/Winter and Spring/Summer CCA (Figure 2.2). According to the CCA, both of these factors were positively related to phosphatase activity. By contrast, DIN:DOP was positively associated with phosphatase activity during spring and summer months only.

*Microbial Heterotrophic Function across Space.* To determine the relationship between microbial activity and stream position (north-south) and watershed land cover of streams, activity of all seven enzymes was assessed at the eight first order study sites (F1, F2, F3, F4, F5, U1, U2, and U3). Principal component analysis was performed on log-transformed enzyme activities to assess how enzyme activity varied across space.

A plot of the first two principal components with PCA scores plotted by site revealed two distinct trends within the dataset (Figure 2.6). The factor loadings of all seven enzymes were positive for principal component 1 (PC1), suggesting that this axis was representative of overall enzyme activity at individual sites. Two of the urbanized sites, U2 and U3, had the highest principal component 1 scores, and therefore higher overall enzyme activity, while the third urbanized site (U1) had the lowest PC1 scores.

The second principal component axis appears to represent a latitudinal gradient that existed regardless of catchment land cover. The southernmost sites had the lowest principal component 2 (PC2) scores, and were associated with higher levels of  $\beta$ Gase, XYLase, NAGase, and, to a lesser extent,  $\alpha$ Gase. Sites located in the northern portion of the Parkers Creek watershed were associated with higher levels of esterase, phosphatase, and LAPase activity. This trend was related to streamwater conductivity, which is higher in sites, both forested and urbanized, north of the Parkers Creek main stem than in the two forested sites located to the south of the Parkers Creek watershed (Table 2.3).

Repeated-measures linear regression showed a significant, positive correlation between stream water conductivity and principal component 2 (PC2) scores (Fig 5b;  $F_{(1,59)}=14.48$ ,  $p<0.001$ ).

To uncover which stream water solutes are responsible for a north-south trend in conductance and microbial function, the concentrations of a number of ion species ( $\text{SO}_4^{2-}$ ,  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{K}^+$ , and total iron) were analyzed for a subset of stream water samples. Conductivity was most strongly correlated to  $\text{Na}^+$  ( $p<0.001$ ), and  $\text{Cl}^-$  ( $p<0.01$ ) as

well as  $Mg^{2+}$  ( $p < 0.001$ ) with significant relationships also found for  $Ca^{2+}$ ,  $K^+$ ,  $Sr^{2+}$  (Table 2.6). By contrast, the gradient in microbial heterotrophic activity, as measured by PC2 scores, was most strongly correlated to sulfate concentrations (Fig 5c,  $F_{(1,37)} = 4.14$ ,  $p < 0.05$ ). With the exception of site U3, which drained a stormwater pond, streams drained directly from artesian springs located within 100 meters of the sampling site (with the exception of site U3, which drained a stormwater pond), indicating that shifts in conductivity are being driven by groundwater geochemistry.

## **Discussion**

Contrary to my hypothesis, there was not a strong and consistent enzymatic response to land cover urbanization. Instead, non-linear temperature responses for EEA resulted in complex responses to changes from urbanization. Seasonal patterns of EEA by site were consistent across two years, as hypothesized, however the patterns of enzyme activity were not as expected. For several enzymes, activity decreased or plateaued at higher temperatures (Figure 2.4). A spike in activity was observed for  $\alpha$ Gase,  $\beta$ Gase, and XYLase during fall sampling events, but activity of these three enzymes was not related to measures of resource availability and quality (e.g. particulate and dissolved C:N, fluorescence index, and dissolved N:P). Instead, activity levels for these three enzymes were highest at moderate temperatures.

*Temperature Impacts a Broad Range of Enzyme Activities.* The environmental factor most significantly related to stream water EEA was temperature and this was true for all enzyme groups except LAPase. While processing rates of individual enzyme molecules

are well known to be influenced by temperature (Chróst, 1991; Sinsabaugh and Shah, 2010), the present experiments were always carried out at 20 °C to ensure comparability across dates. Thus, the Parkers Creek results presented here are indicative of changes in microbial production of extracellular enzymes with temperature, rather than temperature dependence of individual enzymes.

Soil EEA decreases above certain optimal temperatures (Trasar-Cepeda et al., 2007); however, a similar pattern for stream EEA has not been previously reported. Here, at lower temperatures, enzyme production was positively related to temperature for all enzymes except LAPase. Microbial metabolic activity and temperature have long been known to be positively related (Raich and Schlesinger, 1992), thus this finding was anticipated. Taking advantage of the well studied nature of enzyme kinetics, metabolic scaling theory has been applied to explain temperature dependence of microbial metabolic activity and extracellular enzyme activity (Sinsabaugh and Shah, 2010). Here, enzyme production was exponentially related to temperature, producing relationships that could be described with the Arrhenius equation, as has been done for other metabolic processes (Gillooly et al., 2001).

At higher temperatures, the relationship between temperature and enzyme activity was inconsistent. As temperature increased, several enzyme activities became unrelated ( $\alpha$ Gase, NAGase) or negatively related ( $\beta$ Gase, XYLase) to temperature (Figure 2.4). The first is that the majority of microbes in these streams function optimally at moderate temperatures. Since the breakpoint temperatures occurred at moderate temperatures

(11.19-14.41 °C; Table 2.4), this may indicate that the microbial communities in these streams are adapted to function best in this range. Mean stream water temperature for each site across all sampling dates spanned a similar range, 9.8-15.8 °C (Table 2.1), providing circumstantial evidence that microbial communities in these streams are adapted for temperatures in this range. A second hypothesis to explain the nonlinear relationship between EEA and temperature is a change in substrate availability across season (Sinsabaugh and Shah, 2010); however, nutrient levels and carbon quality did not change meaningfully across season.

The strong overall temperature effects I found and the temperatures predicted for streams in the region under future climates (van Vliet et al., 2011) suggest the potential for significant changes in both microbial activity and biogeochemical processing rates for these streams. Changes may be particularly pronounced in urban streams since urbanization and climate change have interactive effects that increase stream temperatures (Nelson et al., 2009) with particularly high temperature spikes in urban streams during rain events (Nelson and Palmer, 2007).

*Placing temperature dependence in context.* Measures of  $Q_{10}$  and  $E_a$  are increasingly being used to determine the relative importance of temperature dependence of microbial metabolic processes, including EEA (Sinsabaugh and Follstad Shah, 2012; Steinweg et al., 2013). In this study I found that enzymes responsible for breaking down carbohydrate resources like  $\alpha$ Gase,  $\beta$ Gase, XYLase were more responsive to temperature than enzymes targeting other resources (Table 2.5). To understand how temperature dependence of

EEA in Parkers Creek compares to other systems, I reviewed values of  $E_a$  from other studies (Figure 2.7, Appendix 2). Studies that report temperature dependence as  $Q_{10}$  were converted to  $E_a$  using equation 3. Due to limited data availability, results were collected from a variety of systems (soil, river sediment, river water column, and stream sediment). I found that estimates of enzyme activity apparent  $E_a$  in soils are much more constrained than freshwater environments, particularly for  $\beta$ Gase (Figure 2.7). The two studies from stream systems (Ylla et al., 2014) reported higher temperature dependence for both  $\beta$ Gase and APase than studies of EEA in soils. Enzyme  $E_a$  in the environment is indicative of increasing system thermodynamic complexity – the number of reactions required to yield a certain amount of energy (Davidson and Janssens, 2006; Sinsabaugh and Follstad Shah, 2012). Thus, higher enzyme  $E_a$  values in freshwaters compared to soils may indicate that different organic matter pools are used in these types of system.

*Weak impact of land cover on EEA.* I hypothesized that EEA would differ between forested and urbanized streams, but only APase activity was significantly greater in urbanized streams across all seasons. This adds to the conflicting results comparing watershed urbanization to EEA. Contrary to my results, other studies that have linked  $\beta$ Gase, LAPase, esterase, and XYLase, in addition to APase, to catchment urbanization (Harbott and Grace, 2005; Williams et al., 2011; Hill et al., 2012; Lehto and Hill, 2013).

The reason for this difference may lie in the degree of landscape urbanization. The relationship between EEA and impervious cover appears to be non-linear. A moderate amount of impervious cover is positively related to enzyme activities at sites U2 and U3.

Site U1 has the highest impervious cover of any site studied (44.0%) and also showed the lowest overall EEA rates (Figure 2.2). There is evidence that lower microbial functional rates with high impervious cover is due to sediment scouring during storm events (Walsh et al., 2005).

Enzyme activity differences between land cover types were driven by environmental factors beyond nutrient stoichiometry. Surprisingly, no significant relationship between phosphatase activity and N:P ratios was found, which contrasts with other studies of stream EEA (Sinsabaugh et al., 2009; Hill et al., 2010a, 2012). Instead, among the factors identified by CCA as being related to phosphatase activity, only fluorescence index was significantly correlated to phosphatase activity ( $F_{(1,52)}=21.37$ ,  $p<0.001$ , Figure 2.5). In these watersheds, increasing fluorescence index values, which are indicative of increasingly autochthonous and labile DOM, are positively related to watershed impervious cover (see Chapter 1). Increased phosphatase levels have long been recognized as an indicator of phosphorus limitation in freshwater primary producers (Pettersson, 1980), suggesting that heightened algal productivity in more eutrophic urban streams may have led to increased demand for sources of phosphorus.

*Groundwater Geochemistry and Enzyme Activity.* Perhaps the most unexpected finding was the apparent influence of groundwater geochemistry on EEA. The south to north transition of enzyme activity coincided with an increase in stream water conductivity; a relationship between EEA and conductivity has been reported in streams before (e.g. Romani and Sabater 2000, Lehto and Hill 2013, Vilches et al 2013). Stream water ion

chemistry is similar to that of data reported for the Magothy aquifer, which exhibits positive potentiometric head across the Parkers Creek study watershed (Knobel and Phillips, 1988) and is likely the source of stream water in this system. The change in conductivity appears to be driven by natural variations in groundwater geochemistry of the Magothy aquifer, which exhibits geochemical gradients running approximately from south to north (Knobel and Phillips, 1988).

The enzymes that were most dominant in streams to the south of Parkers Creek ( $\alpha$ Gase,  $\beta$ Gase, NAGase, and XYLase) all exhibited substantially higher  $Q_{10}$  values than enzymes APase and esterase, which were found in greater concentrations in the northern streams (Table 2.3). This suggests that geochemistry, or another factor related to the north-south EEA gradient in the Parkers Creek watershed, can alter the temperature response of microbial metabolism.

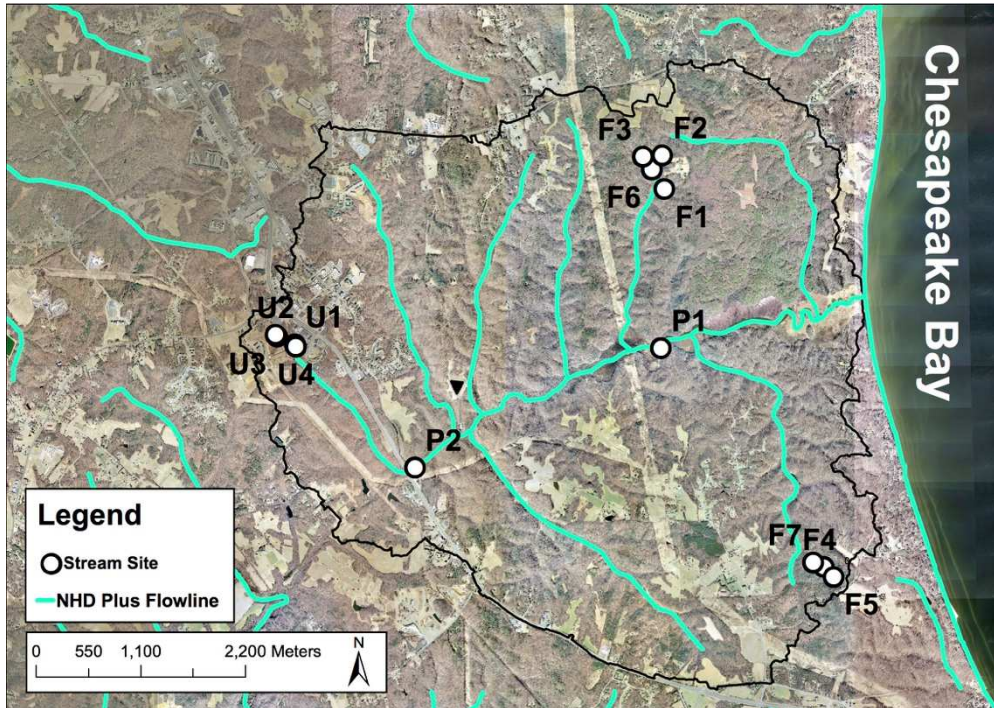
*Complex controls on microbial metabolism.* My results indicate that heterotrophic activity in headwater stream microbial communities is controlled by a diverse set of environmental controls. Rather than being driven primarily by nutrient stoichiometry and straightforward relationships with temperature, this study found more complex relationships. Studies over broad scales have shown that microbial nutrient stoichiometry is central to regulating EEA levels, but also that only so much variability can be explained by nutrients alone (Sinsabaugh et al., 2009; Hill et al., 2010a). At the scale of a small watershed, it appears that the effect of nutrient stoichiometry can be overwhelmed by other factors. As a result, EEA in Parkers Creek headwater streams was better



explained by complicated temperature relationships and changes in stream water conductivity than nutrient stoichiometry.

Efforts to map microbial heterotrophic function in streams are challenging and data-intensive, however given the important role that headwaters play in the carbon cycle, such knowledge is critical. With increasing analytical power and decreasing costs of many molecular analytical methods, there is an opportunity to develop the datasets necessary to uncover the most important factors driving stream metabolic activity. In addition to increasing researchers' ability to map and refine carbon fluxes, a better understanding of microbial enzyme activities can help address fundamental ecological questions regarding the controls on microbial community metabolism and structure. Such insights have the potential to not only inform ecological science as a whole, but also improve the power to predict microbial functional impacts across ecosystems.

## Tables and Figures



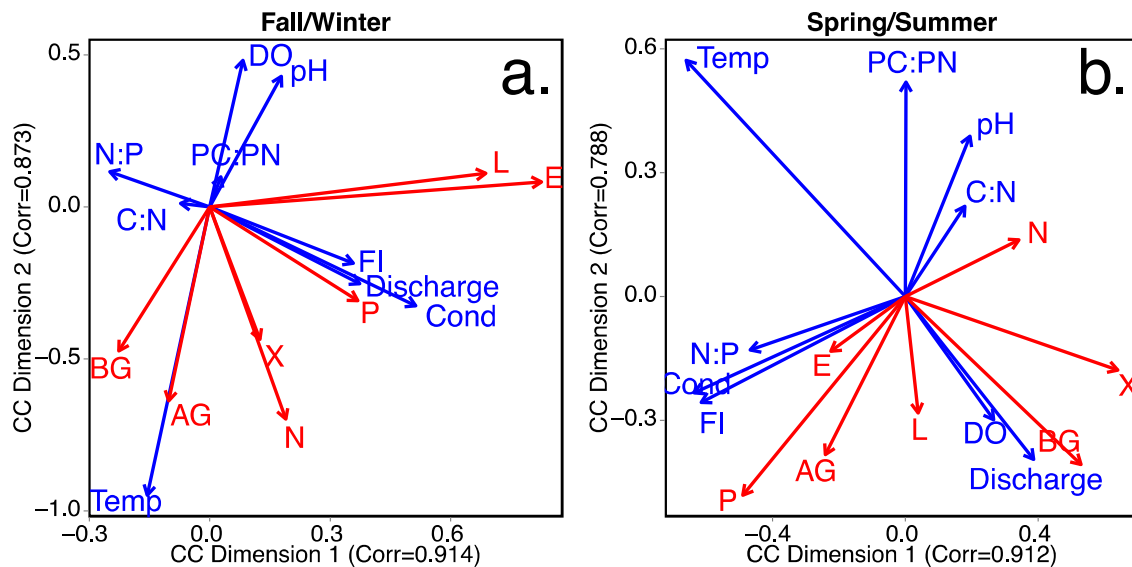
**Figure 2.1:** A map of the Parkers Creek watershed with the study sites indicated. Some stream sampling sites are located in channels that are not included in NHD Plus maps. Aerial orthophotography was provided by Calvert County, Maryland government.

**Table 2.1:** Summary of site identities, watershed area, and location.

<b>Site</b>	<b>Primary Land Cover</b>	<b>Stream Reach Order</b>	<b>Watershed Area (Hectares)</b>	<b>Latitude</b>	<b>Longitude</b>	<b>% Forest Cover</b>	<b>% Impervious Cover</b>
<b>F1</b>	Forested	First	11.70	38°32'51.31"N	76°32'28.97"W	90.6%	0.4%
<b>F2</b>	Forested	First	4.413	38°33'01.94"N	76°32'30.23"W	64.4%	4.9%
<b>F3</b>	Forested	First	6.970	38°33'01.62"N	76°32'39.14"W	94.7%	2.1%
<b>F4</b>	Forested	First	2.855	38°30'41.87"N	76°31'21.21"W	100%	0.0%
<b>F5</b>	Forested	First	2.778	38°30'38.71"N	76°31'16.67"W	90.8%	2.0%
<b>U1</b>	Urbanized	First	1.530	38°31'58.57"N	76°35'09.51"W	33.5%	44.0%
<b>U2</b>	Urbanized	First	7.708	38°32'01.84"N	76°35'18.19"W	27.7%	24.3%
<b>U3</b>	Urbanized	First	4.693	38°32'00.43"N	76°35'17.90"W	2.5%	10.0%
<b>F7</b>	Forested	Second	16.51	38°30'44.08"N	76°31'25.32"W	93.0%	2.5%
<b>F6</b>	Forested	Second	21.38	38°32'57.27"N	76°32'35.09"W	86.5%	1.7%
<b>U4</b>	Urbanized	Second	27.51	38°31'57.68"N	76°35'09.02"W	24.2%	24.1%
<b>P2</b>	Mixed	Third	336.7	38°31'16.68"N	76°34'18.48"W	56.8%	20.0%
<b>P1</b>	Mixed	Fourth	2181.7	38°31'56.56"N	76°32'31.41"W	74.6%	7.52%

**Table 2.2:** A list of the enzymes and corresponding substrates assayed in this study. The environmental compounds that each enzyme group likely degrades are also identified. Table based on previous studies: (Sinsabaugh et al., 1991), (Harbott and Grace, 2005), (Freimann et al., 2013) (Findlay, Stuart, 2007).

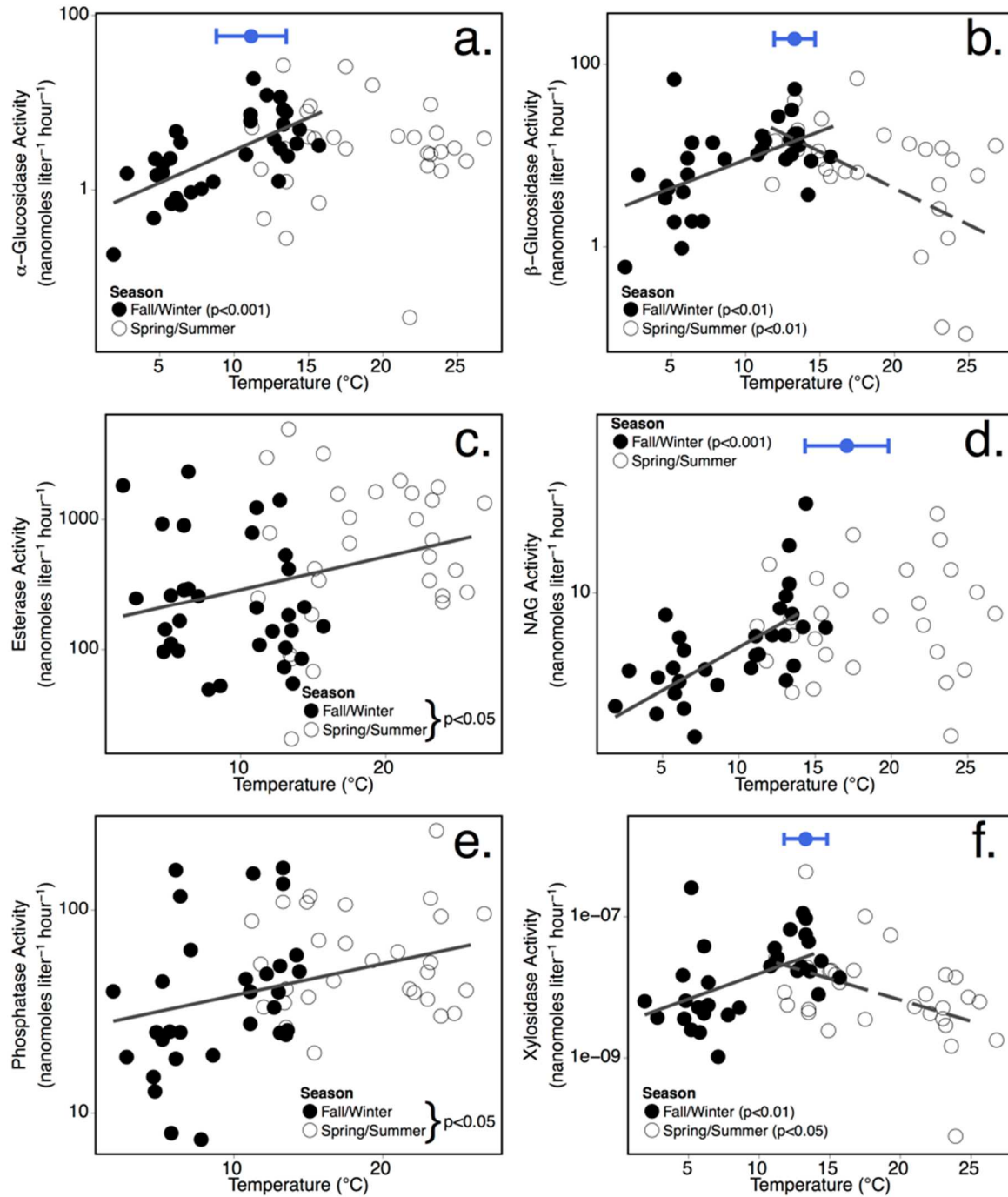
Enzyme	Substrate	Nutrients Acquired	Substrates Degraded
$\alpha$ -Glucosidase ( $\alpha$ Gase)	4-MUB- $\alpha$ -D-glucoside	Carbon	Starch
$\beta$ -Glucosidase ( $\beta$ Gase)	4-MUB- $\beta$ -D-glucoside	Carbon	Cellulose
$\beta$ -Xylosidase (XYLase)	4-MUB- $\beta$ -D-xylopyranoside	Carbon	Hemicellulose
Esterase	4-MUB-Acetate	Carbon	Glycerides
Leucine-Aminopeptidase (LAPase)	L-leucine-7-amido-4-methylcoumarin	Nitrogen	Peptides
N-acetyl-glucosaminidase (NAGase)	4-MUB-N-acetyl- $\beta$ -D-glucosaminide	Nitrogen	Chitin, peptidoglycans, lipopolysaccharides
Alkaline Phosphatase (APase)	4-MUB-Phosphate	Phosphorous	Proteins, nucleotides, organic phosphates



**Figure 2.2:** Results of CCA comparing enzyme activities (red) to environmental factors (blue) in stream water samples during (a) fall and winter and (b) spring and summer. Extracellular enzymes included in analysis are alpha-glucosidase (AG), beta-glucosidase (BG), esterase (E), leucine aminopeptidase (L), N-acetyl-glucoasminidase (NAG), phosphatase (P), xylosidase (X). Environmental factors include conductivity (Cond), discharge (Dis), dissolved oxygen (DO), fluorescence index (FI), DOC:TDN (C:N), PC:PN, DIN:ORP (N:P), pH, temperature (Temp). Significance of canonical correlations was calculated with Bartlett's  $\chi^2$  test. In Fall/Winter both the first ( $\chi^2=135.2$ ,  $df=63$ ,  $p<0.001$ ) and second ( $\chi^2=93.90$ ,  $df=48$ ,  $p<0.001$ ) canonical correlations were significant. In Spring/Summer the first canonical correlation was significant ( $\chi^2=82.84$ ,  $df=63$ ,  $p<0.05$ ), but the second was not ( $\chi^2=44.47$ ,  $df=48$ ,  $p=0.62$ ).

Table 2.3: Summary statistics for variables used in CCA. Mean values by site are reported with standard error of the mean in parenthesis.

Site	Order	Landcover	% Impervious Cover	% Forest Cover	Mean Spring/Summer Temperature (°C)	Mean Fall/Winter Temperature (°C)	Mean Temperature (°C)	Spec. Conductance (µS/cm)	pH	DO Concentration (mg/L)	FI	Discharge (L/s)	DIN:Ortho-P	DOC:TDN	PC:PN
F1	First	Forested	0.40	90.6	13.4 (1.6)	8.0 (2.4)	9.82 (1.96)	265 (33.5)	7.0 (0.16)	6.14 (1.03)	1.45 (0.012)	0.72 (0.01)	2.71 (1.98)	24.7 (3.08)	19.5 (2.07)
F6	Second	Forested	1.71	86.5	17.2 (2.5)	9.0 (2.7)	12.7 (2.27)	147 (14.3)	7.2 (0.14)	7.02 (1.19)	1.41 (0.024)	1.92 (0.02)	4.21 (1.21)	14.7 (1.85)	19.5 (0.21)
F2	First	Forested	4.95	64.4	18.0 (2.8)	8.3 (2.4)	13.2 (2.51)	132 (9.80)	6.7 (0.14)	5.09 (0.73)	1.34 (0.008)	0.39 (0.01)	7.15 (1.34)	30.3 (8.05)	19.4 (3.04)
F3	First	Forested	2.06	94.7	17.6 (2.3)	8.7 (2.0)	13.1 (2.20)	157 (24.1)	6.8 (0.11)	6.85 (0.77)	1.41 (0.004)	0.60 (0)	7.97 (1.74)	11.4 (1.74)	19.6 (1.51)
F4	First	Forested	0.00	100	17.5 (4.0)	9.5 (2.0)	12.9 (2.47)	49.3 (1.86)	6.7 (0.16)	6.40 (1.56)	1.33 (0.011)	0.26 (0.05)	3.5 (1.11)	31.2 (5.5)	17.9 (1.26)
F5	First	Forested	2.02	90.8	18.5 (2.9)	10.2 (1.9)	14.3 (2.24)	57.4 (2.82)	6.5 (0.19)	5.33 (1.27)	1.34 (0.014)	0.20 (0.06)	9.64 (2.52)	27.8 (3.94)	16.5 (1.03)
F7	Second	Forested	2.02	93.0	18.8 (3.3)	8.3 (3.8)	14.2 (2.50)	131 (11.4)	6.9 (0.13)	5.67 (1.24)	1.36 (0.011)	0.94 (0.31)	10.1 (1.67)	11.8 (1.15)	14.7 (2.64)
U4	Second	Urbanized	24.1	24.2	21.0 (2.3)	9.1 (4.8)	15.5 (3.10)	535 (34.1)	7.3 (0.49)	5.95 (1.40)	1.5 (0.007)	3.26 (0.7)	32.5 (9.79)	8.96 (2.74)	16.5 (0.94)
U1	First	Urbanized	44.0	33.5	19.6 (2.2)	8.9 (2.3)	14.3 (2.52)	254 (24.0)	6.6 (0.10)	5.92 (0.98)	1.59 (0.008)	0.35 (0.09)	237 (54.7)	4.60 (1.59)	16.1 (0.89)
U2	First	Urbanized	24.3	27.7	20.5 (1.7)	10.0 (2.3)	15.2 (2.37)	254 (24.2)	6.5 (0.10)	5.65 (1.04)	1.58 (0.009)	0.84 (0.23)	107 (56.4)	5.37 (1.28)	14.7 (3.34)
U3	First	Urbanized	10.0	2.50	21.5 (2.7)	10.4 (2.4)	15.9 (2.68)	106 (4.10)	6.7 (0.13)	4.94 (1.26)	1.54 (0.009)	0.97 (0.28)	29.8 (16.38)	11.3 (4.3)	11.3 (4.30)



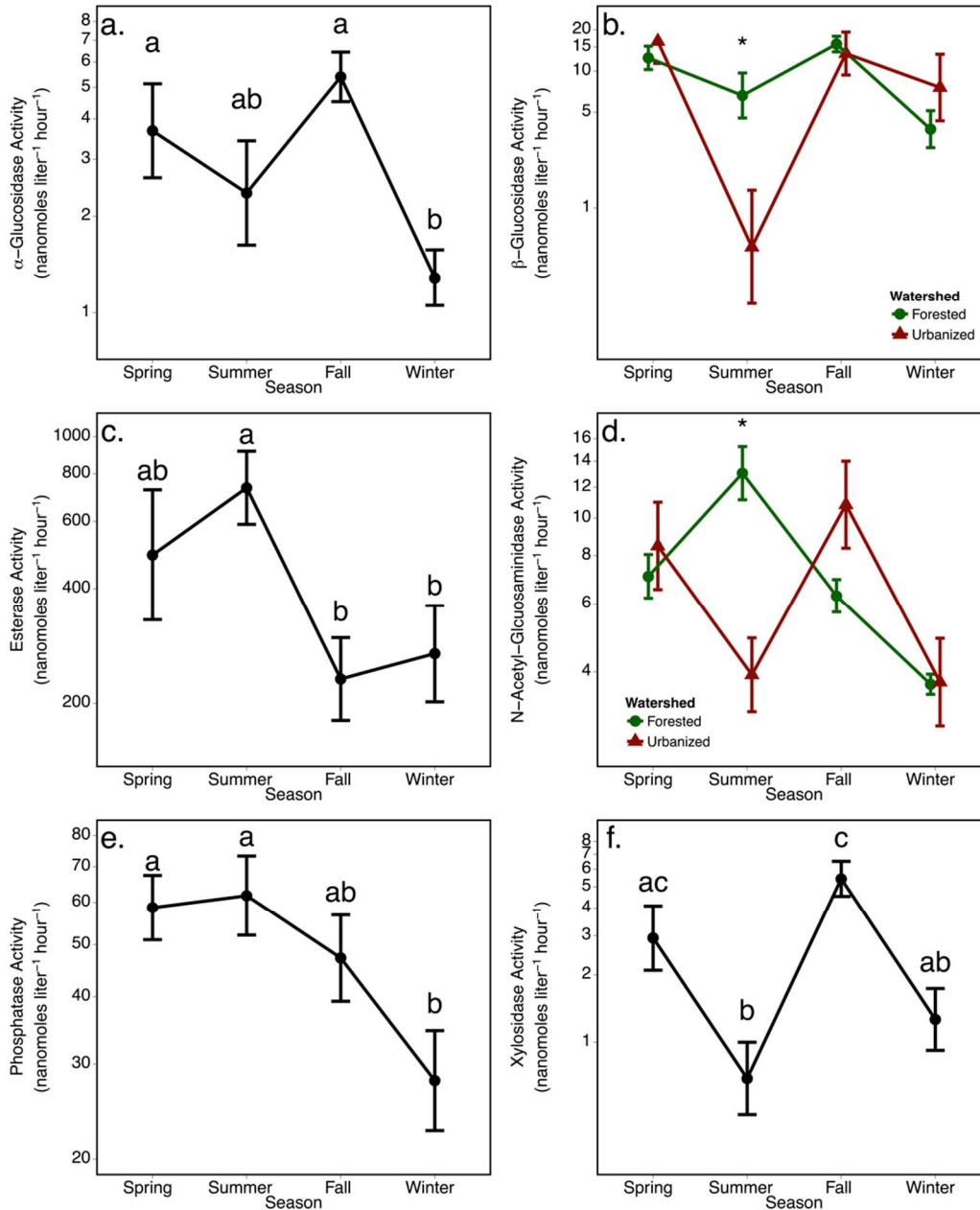
**Figure 2.3:** Temperature compared to (a) alpha-glucosidase, (b) beta-glucosidase, (c) esterase, (d) N-acetyl-glucosaminidase, (e) phosphatase, and (f) xylosidase activity of stream water with season (“Fall/Winter” or “Spring/Summer”) included as a covariate. Significance tests were conducted using repeated-measures ANCOVA. Significant seasonal interactions were found for alpha-glucosidase (p<0.01), beta-glucosidase (p<0.001), N-acetyl-glucosaminidase (p<0.001), and xylosidase (p<0.001) enzyme activities. For enzymes with significant seasonal interactions, breakpoints were determined with piecewise regression. Significant breakpoints are indicated with blue dots; error bars indicate standard error of breakpoint estimates.

**Table 2.4:** For enzymes that showed a significant interaction between Spring/Summer and Fall/Winter, the breakpoint temperature where the relationship between enzyme activity and temperature changed was determined using piecewise regression modeling. P-values test whether the change in regression slope above and below the breakpoint is significant. Analysis was conducted on log-transformed enzyme activities.

<b>Enzyme</b>	<b>Breakpoint (°C)</b>	<b>Breakpoint Standard Error</b>	<b>p-value</b>
$\alpha$ Gase	11.19	2.35	0.035
$\beta$ Gase	13.29	1.37	0.012
NAGase	14.41	2.35	0.00024
XYLase	13.27	1.52	0.022

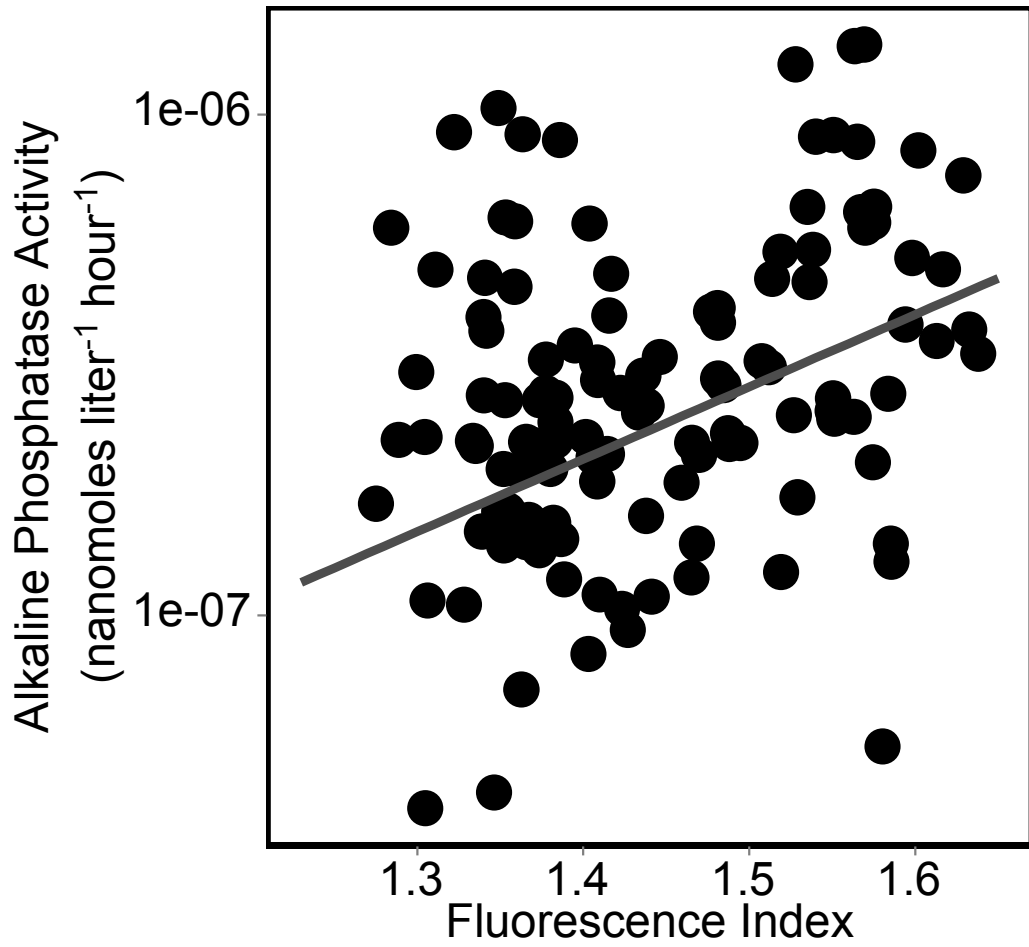
**Table 2.5:**  $Q_{10}$  values for each of the six enzyme classes assayed for which there was a significant relationship with temperature.

<b>Enzyme</b>	<b><math>Q_{10}</math></b>
$\alpha$ Gase	5.57
$\beta$ Gase	4.19
Esterase	1.80
NAGase	2.57
APase	1.43
XYLase	5.27

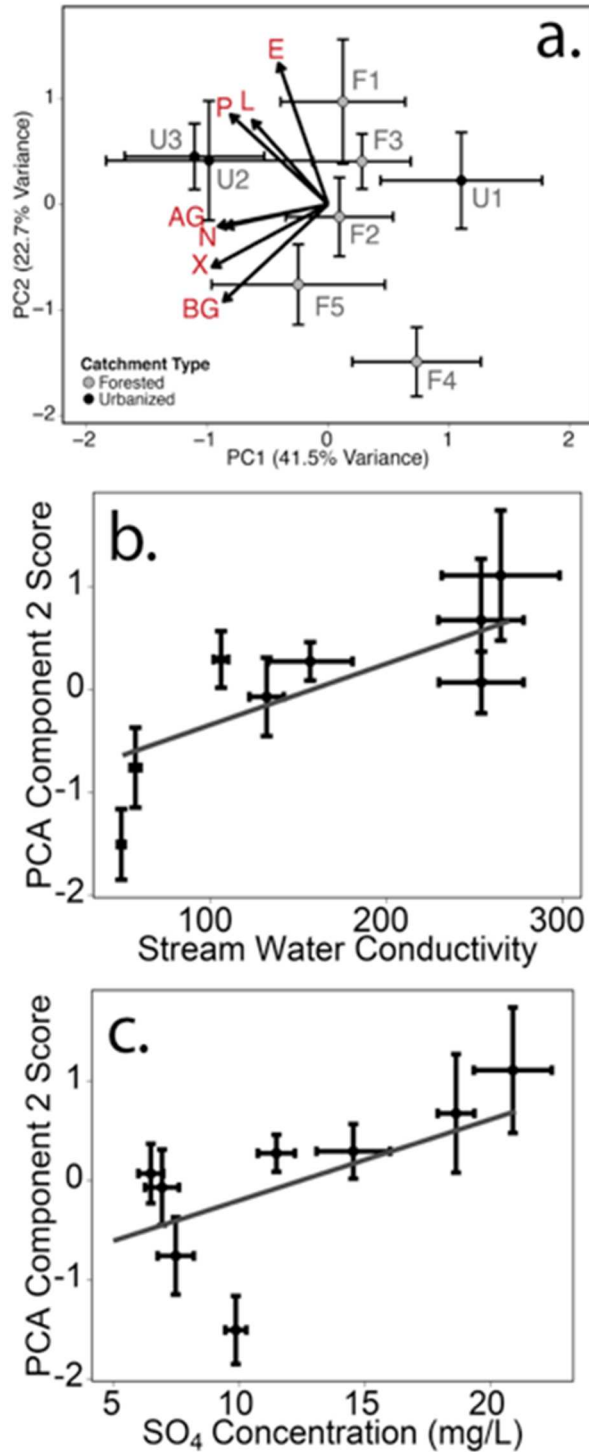


**Figure 2.4:** Activity by season (spring, summer, fall, and winter) for (a) alpha-glucosidase, (b) beta-glucosidase, (c) esterase, (d) N-acetyl-glucosaminidase, (e) phosphatase, and (f) xylosidase enzymes. Statistical tests of main effects and interactions were conducted with repeated-measures ANOVA. Multiple mean comparisons were conducted with Tukey's HSD. Different letters indicate a statistically significant difference between groups across sampling season. An asterisk indicates statistically significant differences between watershed types for one sampling season. All statistical tests were applied with an alpha of 0.05.

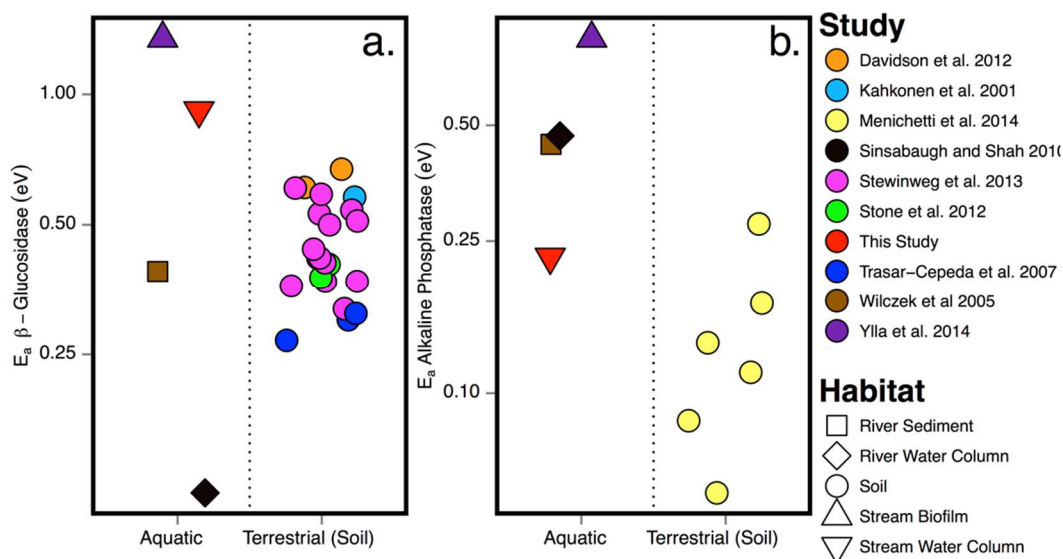




**Figure 2.5:** Fluorescence Index compared to Phosphatase activity in eight first order streams sites. The positive relationship between FI and phosphatase activity was significant as measured by repeated-measures ANCOVA ( $p < 0.001$ ).



**Figure 2.6:** Overall extracellular enzyme activity was analyzed by site via principal component analysis (a). A significant relationship between PCA component 2 scores and (b) stream water conductivity and (c) sulfate concentrations was identified. Error bars represent standard error of the mean calculated by stream sampling site. Analysis was conducted on first order stream sites exclusively.



**Figure 2.7:** Results of a meta-analysis comparing  $E_a$  values collected from microbial EEA in freshwater and soil systems. Each point represents  $E_a$  estimates from one system and the environment in which the study was conducted is identified. Activation energy values are reported in electron volts (eV) for a)  $\beta$ -glucosidase and b) alkaline phosphatase. Appendix II contains a table of values is presented in this figure.

**Table 2.6:** Repeated Measures ANOVAs comparing streamwater conductivity to the concentrations of several common ions.

Independent Variable	Dependent Variable	Num DF	Den DF	Fvalue	P
Na <sup>+</sup>	Conductivity	1	17	31.88	<0.0001
Ca <sup>2+</sup>	Conductivity	1	17	5.00	0.039
K <sup>+</sup>	Conductivity	1	17	7.02	0.017
Si <sup>2+</sup>	Conductivity	1	17	5.75	0.028
Mg <sup>2+</sup>	Conductivity	1	17	18.21	<0.001
SO <sub>4</sub> <sup>2-</sup>	Conductivity	1	32	0.00	0.972
Cl <sup>-</sup>	Conductivity	1	32	15.82	<0.001
Total Fe	Conductivity	1	28	0.25	0.624

**Table 2.7** – A review of studies comparing stream EEA to environmental variables. The variables that have been positively or negatively related to commonly analyzed enzyme groups are reported

Enzyme	Positively Related Variables	Negatively Related Variables
<b><math>\alpha</math>-glucosidase/<math>\beta</math>-glucosidase</b>	<ul style="list-style-type: none"> <li>Chlorophyll (Smucker and Vis 2011‡, Artigas et al 2012‡, Romani and Sabater 2000‡)</li> <li>Imperviousness (Williams et al 2011*)</li> <li>Light (Rier et al 2014‡)</li> <li>DOC (Romani and Sabater 2000‡)</li> <li>DIC (Romani and Sabater 2000‡)</li> <li>Conductivity (Romani and Sabater 2000‡)</li> <li>Temperature (Romani and Sabater 2001†, this study*)</li> <li>Ammonium (Romani and Sabater 2001†), TDN (Williams et al 2011*)</li> <li>TDP (Williams et al 2011*)</li> <li>TSS (Williams et al 2011*)</li> </ul>	<ul style="list-style-type: none"> <li>Oxygen (Romani and Sabater 2001†)</li> <li>pH (Simon et al 2009◊)</li> <li>Temperature (This study*)</li> <li>Conductivity (This study*)</li> </ul>
<b>Leucine-aminopeptidase</b> (except where noted below)	<ul style="list-style-type: none"> <li>Chlorophyll (Artigas et al 2012‡)</li> <li>Effective Imperviousness (Harbott and Grace 2005*)</li> <li>DOC (Harbott and Grace 2005*)</li> <li>pH (Smucker and Vis 2011‡)</li> <li>Catchment forest cover (Hill et al 2012‡,†)</li> <li>Light (Rier et al 2014‡)</li> <li>TDP (Williams et al 2011*)</li> <li>Conductivity (This study*)</li> </ul>	<ul style="list-style-type: none"> <li>Imperviousness (Lehto and Hill 2013‡)</li> <li>Chloride (Lehto and Hill 2013‡)</li> <li>DOC (Hill et al 2012‡,†; Williams et al 2011*)</li> <li>TN (Hill et al 2012†)</li> </ul>
<b>Esterase</b>	<ul style="list-style-type: none"> <li>Effective Imperviousness (Harbott and Grace 2005*)</li> <li>DOC (Harbott and Grace 2005*)</li> <li>Temperature (This study*)</li> <li>Watershed Size (This study*)</li> <li>Conductivity (This study*)</li> </ul>	
<b><math>\beta</math>-xylosidase</b>	<ul style="list-style-type: none"> <li>Chlorophyll (Romani and Sabater 2000‡)</li> <li>DOC (Romani and Sabater 2000‡)</li> <li>DIC (Romani and Sabater 2000‡)</li> <li>Conductivity (Romani and Sabater 2000†)</li> <li>Temperature (Romani and Sabater 2001†, this study*)</li> <li>Light (Rier et al 2014‡)</li> <li>TP (Rier et al 2014‡)</li> </ul>	<ul style="list-style-type: none"> <li>Effective Imperviousness (Harbott and Grace 2005*)</li> <li>pH (Simon et al 2009◊)</li> <li>Chlorophyll (Smucker and Vis 2011‡)</li> <li>Oxygen (Romani and Sabater 2001†)</li> <li>Ammonium (Romani and Sabater 2001†)</li> <li>Temperature (This study*)</li> </ul>
<b>Alkaline Phosphatase</b> (except where noted below)	<ul style="list-style-type: none"> <li>TP (Rier et al 2011‡), TDP (Williams et al 2011*)</li> <li>pH (Vilches et al 2013‡)</li> <li>Temperature (This study*)</li> <li>Imperviousness (Williams et al 2011*)</li> <li>Conductivity (Vilches et al 2013‡)</li> <li>Catchment forest cover (Hill et al 2012‡,†)</li> <li>Light (Romani and Sabater 2001‡; Rier et al 2014‡)</li> <li>TSS (Williams et al 2011*)</li> </ul>	<ul style="list-style-type: none"> <li>Imperviousness (Lehto and Hill 2013‡)</li> <li>Chloride (Lehto and Hill 2013‡), Conductivity (This study*)</li> <li>pH (Simon et al 2009◊, Smucker and Vis 2011‡)</li> <li>SRP (Vilches et al 2013‡), TP (Hill et al 2012‡,†)</li> <li>TN (Hill et al 2012‡,†)</li> </ul>

Sample type: \* Water, ‡Biofilm, †Sediment, §Periphyton, ◊Leaf Litter. Smucker and Vis (2011) report activities as phosphatase:leucine-aminopeptidase and  $\beta$ -glucosidase:  $\beta$ -xylosidase. Lehto and Hill (2013) report total peptidase activity. Hill et al (2012) report peptidase activity as the combination of l-alanine aminopeptidase, l-leucine aminopeptidase, beta-N-acetylglucosaminidase and phosphatase as acid phosphatase. Rier et al (2014) used shade manipulation and nutrient diffusing substrates to determine light and phosphorus effects. Williams et al (2011) report enzyme activities of whole water samples. TDN = Total dissolved nitrogen, DIC = dissolved inorganic carbon, TDP = total dissolved phosphorus, TSS = total suspended solids, SRP = soluble reactive phosphorus, TP = total phosphorus, TN = total nitrogen.

## Chapter 3: Headwater stream bacterial diversity reveals significant influence of urbanization and habitat on community structure.

### **Introduction**

Despite the importance of microbes to food webs and biogeochemical processes in freshwater systems, the controls on microbial diversity and community composition in streams remain poorly understood. Several studies suggest that microbial community structure in streams, particularly in bed sediments, is strongly tied to stream sampling site (Hullar et al., 2006; Or et al., 2012; Portillo et al., 2012); however, the underlying mechanisms for this pattern are unclear. Microbial community composition is related to both stream site (e.g., (Crump et al., 2003, 2003; Judd et al., 2006, 2; Beier et al., 2008; Besemer et al., 2013; Zeglin et al., 2011, 20; Fierer et al., 2007) and watershed scale environmental factors such as catchment geology (Larouche et al., 2012). Further, dispersal (Crump et al., 2007, 2007; Beier et al., 2008; Crump et al., 2012), plays an important role as well. Comparing bacterial community composition across watershed environmental gradients, such as impervious cover, can help identify key factors structuring communities.

Alterations to microbial community functions in stream by urbanization are widely recognized. Increased nutrient flows often result in greater algal and bacterial metabolic rates and biomass in streams as a result of urbanization though levels can drop if adverse factors like scouring and heavy metal exposure are high (Walsh et al., 2005; Catford et al., 2007; Imberger et al., 2008). Urbanization also alters rates of nutrient retention

(Mulholland et al., 2001; Meyer et al., 2005; Mulholland et al., 2008) and denitrification (Groffman and Crawford, 2003; Harrison et al., 2012). What is less well understood is whether changes in urbanized stream microbial function are the result of an altered microbial community or simply the same microbes responding to new environmental conditions. To begin to answer this question, the fundamental relationships between urbanized land use and microbial community structure must first be determined.

Research on the effects of urbanization on bacterial communities is largely limited to studies of pathogenic taxa such as fecal coliform bacteria (Nagy et al., 2012; Duris et al., 2013; Daly et al., 2013; Liang et al., 2013; Line, 2013; McGrane et al., 2014; Kapoor et al., 2014), denitrifying bacteria (Hale and Groffman, 2006; Knapp et al., 2009; Harrison et al., 2012), and unicellular algae and diatoms (Hill et al., 2000; Fore and Grafe, 2002; Newall and Walsh, 2005; Catford et al., 2007; Elsdon and Limburg, 2008). Research that examines the effect of watershed urbanization on microbial community composition and diversity is limited to a handful of studies. Differences in community composition between urbanized and forested systems has been demonstrated in a variety of habitats (Lear and Lewis, 2009; Lear et al., 2011) including streambed sediments (Jackson and Weeks, 2008; Perryman et al., 2011a; Wang et al., 2011), and water column (Belt et al., 2007; Or et al., 2013). This work provides important insights, but available research relies on coarse measurements of microbial diversity such as fluorescence in-situ hybridization (FISH), automated ribosomal intergenic spacer analysis (ARISA), and terminal restriction fragment length polymorphism (T-RFLP) analysis. Lack of both

analytical depth and sequenced data has limited understanding of what microbial taxa drive differences between forested and urbanized streams.

In headwater streams, bacterial community composition in the water column is largely controlled by the surrounding catchment, which is the source of most bacterial cells via downslope water flow (Crump et al., 2012). Since urbanization alters the composition of the water column microbial community (Belt et al., 2007; Brinkmeyer et al., 2015), this may change which microbial taxa are available to colonize bed sediments. Environmental filtering occurs as microbes in transit through the water column colonize more stable stream bed environments (Araya et al., 2003; Yamaguchi et al., 1997). Stream bed microbial community structure is strongly tied to site and highly resilient to disturbance (Hullar et al., 2006; Or et al., 2012), which may be explained by experimental evidence suggesting a strong influence of environmental sorting on microbial composition (Or et al., 2012; Adams et al., 2014; Handley et al., 2014). Though environmental sorting is an important factor, there is evidence that microbial dispersal impacts community structure as well (Crump et al., 2007; Badin et al., 2012; Crump et al., 2012). Continuous dispersal of watershed microbes through stream networks may influence downstream community structure by exerting a mass effect, changing microbial community composition via import of large numbers of cells from outside the system (Crump et al., 2007).

New technology allows for dramatic increases in the number of microbial sequences obtained in each sample, creating new analytical challenges while also providing new opportunities to uncover linkages among taxa (Barberán et al., 2011). Co-occurrence

network analysis allows researchers to identify microbes that are most central to structuring bacterial assemblages, including potential keystone taxa (Barberán et al., 2011; Williams et al., 2014; Lupatini et al., 2014; Widder et al., 2014). Further, networks derived from different ecosystems can be compared to assess the relative importance of specific microbial interactions across environments (Williams et al., 2014). Modularity and clustering coefficients, network attributes that describe the degree of partitioning within an individual network, may be indicative of environmental heterogeneity and level of disturbance within an environment (Newman, 2006; Parter et al., 2007; Olesen et al., 2007). Thus, study of microbial co-occurrence networks can provide unique insight into the controls on microbial communities.

Given that knowledge gaps exist regarding how watershed urbanization impacts microbial community structure in headwater streams, the objectives of the study were to determine: 1) the relationship between stream watershed type (forested and urbanized) and sample habitat (water column and stream bed sediments) on bacterial community composition; 2) the environmental factors governing community composition; and 3) how microbial relationships change across environments by comparing microbial co-occurrence networks produced from different site types and habitats. I hypothesized that microbial community structure would be related both to sampling habitat (water column, sediment) and stream watershed land cover type (forested, urbanized), with watershed type being the more significant of the two factors. Further, I predicted that environmental differences between forested and urbanized streams would result in differing microbial



co-occurrence networks with urbanization resulting in simplified, less modular networks compared to forested streams.

## **Methods**

*Study sites.* The study was conducted in the Parkers Creek watershed, located on the Western Shore of the Chesapeake Bay in the Coastal Plain of Maryland, USA. The study streams are composed of alluvial materials and represent a gradient of land use (see Chapter 1). Study sites were located in first, second, third, and fifth order reaches within the Parkers Creek watershed. Many of the headwater streams in this watershed are unmapped, therefore determination of stream order was carried out using field observations and watershed orthophotography provided by the Calvert County, Maryland government.

This study reports on a total of 11 individual stream sites (Figure 1, Table 3.1). Five forested first order stream sites were studied (F1, F2, F3, F4, F5) with an additional two sites were located in second order forested reaches (F6 and F7). Three urbanized first order stream sites were identified (U1, U2, and U3), along with one second order site (U4). Additional details on the eight first order reaches (F1, F2, F3, F4, F5, U1, U2, and U3) are presented in Chapter 1.

*Sample collection.* Water column samples were collected in February, April, August, and November 2012 and February 2013 from all sites with surface water present. Sediment samples were collected contemporaneously with water column samples in August and

November 2012 and February 2013. Water column samples were collected following (Crump et al., 2003, 2003). In the field, 300-600 mL of stream water were passed through a Millipore Sterivex-GP 0.22  $\mu\text{m}$  filter. Residual water was expelled from the filter and approximately 2 mL of DNA extraction buffer were added, after which both ports of the filter were sealed. Sediment samples were collected from streambeds to a depth of 3 cm using 2.67 cm diameter sterile plastic coring devices. Twenty cores were taken from random points along a 20-meter reach at each site on each sampling date. All cores taken at a site were combined in a single sterile Nasco Whirlpak bag.

Stream water temperature, specific conductivity, dissolved oxygen, and pH were determined in the field using a YSI Professional Plus multimeter (YSI Inc., Yellow Springs, OH). Stream water samples for chemical analysis were collected in amber borosilicate bottles that had been acid washed and subsequently combusted at 450°C for four hours and were sealed with acid-washed Teflon-coated lids. All samples were placed on ice for transport to the laboratory. Samples for genetic analysis were subsequently stored at -80°C prior to further processing. Samples for chemical analysis were stored at 4°C for less than 48 hours until sample analysis.

*Physicochemical variables.* Streamwater temperature, specific conductivity, pH, and dissolved oxygen were measured in the field using a YSI Professional Plus multiparameter meter (YSI Inc.; Yellow Springs, OH). Samples for dissolved organic carbon (DOC) concentration and DOM quality measurements were filtered in the field with 0.7  $\mu\text{m}$  GF/F filters (Whatman Inc., Maidenstone, UK) and were stored in

borosilicate amber glass bottles with Teflon-coated lids. Sample bottles were acid washed in 10% HCl and both bottles and GF/F filters were combusted for 4 hours at 450°C.

Water for other analysis was filtered in the field into amber HDPE plastic bottles. All samples were returned to the laboratory on ice. Samples for carbon quality were kept at 4 °C and were analyzed within 72 hours of collection. Other samples were frozen until processing at a later date.

Total DOC, as non-purgeable organic carbon, and total dissolved nitrogen (TDN) were determined by analysis on a Shimadzu TOC-vCPH with attached TNM-1 unit (Shimadzu Corporation; Kyoto, Japan). Carbon quality was determined using the fluorescence index (FI), which is an indicator that DOM in a water sample is primarily allochthonous or autochthonous (McKnight et al., 2001). The fluorescence index was determined as the ratio of fluorescence emission intensities at 450 and 500 nm when a water sample was excited at 370 nm (McKnight et al., 2001). Humification index (HIX) values were determined as the ratio of the area of the emission spectrum at 435 to 480 nm to the emission area from 300 to 445 nm at an excitation wavelength of 255 nm (Zsolnay et al., 1999; Plaza et al., 2009; Williams et al., 2010). Fluorescence data were collected on a Horiba Scientific Fluoromax-4 as described in Chapter 1.

Dissolved inorganic nitrogen was determined as the sum of dissolved ammonium and nitrate. Dissolved organic nitrogen was calculated by subtracting dissolved inorganic nitrogen from total dissolved nitrogen concentrations. A Lachat QuikChem 8500 Series 2

flow injection analyzer was used to find dissolved nitrate, ammonium, and orthophosphate concentrations.

*Sample processing.* Water column microbial DNA was extracted from Sterivex-GP filters using phenol-chloroform based on established protocols (Crump et al., 2003, 2003).

Filters and DNA extraction buffer were defrosted and 20  $\mu$ L of 1% proteinase-K and 20  $\mu$ L of 10% lysozyme was added to each filter. Samples were frozen at  $-80^{\circ}\text{C}$  for 15 minutes and then thawed at  $37^{\circ}\text{C}$  for 5 minutes a total of three times. Samples were then incubated in a water bath for  $37^{\circ}\text{C}$  for 30 minutes. Fifty  $\mu$ L of 20% filter-sterilized sodium dodecyl sulfate (SDS) were added to each sample before a 2 hour incubation in a  $65^{\circ}\text{C}$  water bath. Samples were washed twice with buffered phenol-chloroform-isoamyl alcohol and then precipitated at room temperature overnight by adding isopropyl alcohol at 60% of sample volume. Microbial sediment DNA was extracted using PowerSoil DNA Isolation Kits (Mo Bio Laboratories, Inc., Carlsbad, CA). To account for the high water content of stream sediment samples, 0.5 grams of sediment was used for each extraction.

PCR amplicons were produced using standard methods for high-throughput sequencing (Caporaso et al., 2012). Amplification of 16S rDNA was conducted using forward primer 515f and barcoded reverse primer 806r obtained from the Earth Microbiome Project. For each sample 12  $\mu$ L of UV-sterilized PCR-grade water, 10  $\mu$ L 5-prime HotMasterMix, 1  $\mu$ L 5 mM forward primer, 1  $\mu$ L of 5 mM reverse primer, and 1  $\mu$ L of template DNA were combined in a 96-well PCR plate. Conditions for PCR were as follows: initial denaturation for 3 minutes at  $94^{\circ}\text{C}$  followed by 30 cycles first at  $94^{\circ}\text{C}$  for 0.75 minutes,

50°C for 1 minute, and 72°C for 1.5 minutes. At the conclusion of each PCR run, temperature was held at 72°C for 10 minutes before temperature was reduced to 10°C. Amplicons were quantified with Pico-Green dsDNA quantification kit (Life Technologies; Carlsbad, CA), combined in equimolar quantities, and cleaned using an UltraClean PCR Clean-Up kit (MO BIO Laboratories, Inc; Carlsbad, CA). Illumina MiSeq 2x150 bp sequencing was conducted at Argonne National Laboratory (Lemont, IL).

*Data analysis.* Data were analyzed using the package Quantitative Insights into Microbial Ecology (QIIME). Paired end reads were matched using FLASH (Magoc and Salzberg, 2011). USEARCH 6.1 (Edgar, 2010) was used to identify OTUs at 97% similarity from the Silva 111 database and to identify chimeric sequences. Taxonomy was assigned using the RDP Classifier (Wang et al., 2007) at a threshold of 80%. Sequences were subsequently aligned using PyNAST (Caporaso et al., 2010). Sequences identified as belonging to chloroplasts, mitochondria, and the order Thermales were removed from the dataset as well as any OTU that was not identified taxonomically to at least the class level. Each sample was then rarified to 25,000 sequences.

Microbial species richness was estimated using CatchAll (Bunge et al., 2012) on rarified OTU tables. All subsequent data analysis and plotting, except where noted, were conducted in R version 3.1.2. Beta diversity was analyzed using principal coordinate analysis (PCoA) of dissimilarity matrices calculated between individual samples using Bray-Curtis distances, which were computed using the *vegan* package (Oksanen et al.,

2013). Adonis and Analysis of similarity (ANOSIM) were conducted in the *vegan* package to compute similarity between groups. Permutations for both Adonis and ANOSIM were restricted to reflect the repeated measures of samples over time and the lack of independence between sediment and water column samples. Indicator species analysis is a measure that identifies taxa that are representative of different sample types (Fortunato et al., 2013). Indicator species analysis produces an indicator value for each combination of taxon and indicator group. If an indicator value for a particular indicator group is greater than 0.3 and that taxon has a significant p-value ( $\alpha = 0.05$ ), then that taxon is considered an indicator. Indicator species analysis was conducted using the *indicspecies* package (Caceres and Legendre, 2009) in R 3.1.2.

The relationship between environmental variables and microbial community structure was assessed using the BV-STEP function with 100 restarts, 10 initial variables,  $\rho > 0.95$ , and minimum  $\Delta\rho > 0.001$  based on the parameters described by (Clarke and Ainsworth, 1993). The r-square value of the resulting model was calculated using canonical correspondence analysis (CCA) using the *vegan* package. For this analysis the microbial dataset was divided into two seasons: warm (spring and summer) and cold (fall and winter). The dataset was further divided by habitat (sediment and water column), resulting in four separate models. For water column communities a total of 16 environmental variables – temperature, dissolved oxygen concentration, pH, and log-transformed discharge, DOC concentration, TDN concentration, dissolved orthophosphate concentration, dissolved organic nitrogen concentration, DOM humification index, FI, conductivity, particulate C:N, particulate C concentration, and

particulate N concentration – were supplied to the BV-STEP function. For sediment communities 14 environmental variables – dissolved oxygen concentration, temperature, pH, and log-transformed discharge, DOC concentration, TDN concentration, HIX, FI, conductivity, sediment percent nitrogen, sediment percent carbon, sediment C:N, watershed percent impervious cover, and watershed area – were used.

Repeated-measures analysis of variance (ANOVA), analysis of covariance (ANCOVA), and linear regressions were carried out in SAS 9.3 using the *Proc Mixed* package.

Normality of ANOVA and ANCOVA variables was evaluated by assessing residuals. Variables that deviated substantially from normality were transformed by taking the logarithm of the variable plus one. Compound symmetry covariance structures were assumed for all ANCOVA analysis.

*Landscape Analysis.* A digital elevation model (DEM) of the Parkers Creek watershed was generated from Light Detection and Ranging (LiDAR) data collected in March 2011 and provided by Calvert County, MD Government. Field site watersheds were extracted from the DEM. Impervious, forested, and agricultural land cover were manually delineated using orthophotography collected in March 2011 and provided by Calvert County, MD Government. To obtain stream network distances, DEM accumulation lines were developed between stream channel sampling points. The distance along these flow lines between sites was determined to be stream network distance between sites. Statistics were developed only for sites directly connected by flow. All landscape analysis was conducted using ArcGIS 10.1.

*Co-occurrence networks.* Co-occurrence network analysis of microbial OTU data was applied following existing methods (Barberán et al., 2011; Williams et al., 2014; Lupatini et al., 2014; Widder et al., 2014). To avoid spurious correlations and to aid in the interpretation of results, low abundance taxa that represented less than 0.025% of total sequences were filtered prior to analysis. Pairwise correlations were calculated for each pair of OTUs using Spearman's rank correlation. For a co-occurrence event to be included in the final network, a threshold of  $|\rho| > 0.75$  and  $p < 0.01$  was adopted. To confirm that the network generated was not the product of random correlations, a comparison was made with randomly generated networks following (Lupatini et al., 2014). One thousand random networks with size (i.e., number of nodes and vertices) equal to the network generated from the microbial dataset were produced using the Erdős-Rényi model. Mean clustering coefficient, mean path length, and network modularity were calculated for each randomly generated network and were compared to the values generated from the experimentally derived network. The p-value of rejecting the null hypothesis that the experimental network was obtained at random was calculated as the proportion of values derived from the randomly generated models that were greater than the values obtained from the experiment for each of the three test statistics. For all three statistics the p-value was less than 0.001, indicating the experimental network was not obtained at random.

For each node, network centrality metrics including degree, closeness centrality, and betweenness centrality were calculated. Node degree is defined as the number of vertices



connected to a node. Betweenness centrality is defined as the number of geodesics that pass through a node when all possible geodesics are considered. Closeness centrality is calculated as the inverse of the average length of all the geodesics connecting one node to each other node in the network (Freeman, 1978). These metrics have the potential to identify keystone species within community networks (Williams et al., 2014). Microbial co-occurrence network analysis on simulated communities with known relationships indicate that both node degree and closeness centrality are positively linked to keystone taxa (Berry and Widder, 2014). Network analysis was conducted using R 3.1.2 with the *vegan* and *igraph* (Csardi and Nepusz, 2006) packages. Networks were visualized using the software package Gephi 0.8.2 (Bastian et al., 2009).

Four co-occurrence networks were generated. Each network was based on samples from a different combination of habitat and stream watershed type (i.e., forested/water column, forested/sediment, urbanized/water column, and urbanized/sediment). Differences among network co-occurrence relationships across the four networks generated were tested using a permutation test described by (Williams et al., 2014). Differences in network structures were described by modularity, transitivity, average path length, and average node degree (Newman, 2003, 2006; Barberán et al., 2011). Modularity is a measure of network aggregation that is defined as the number of edges within a network minus the number of edges within a network of equal size that is produced at random (Newman, 2006).

Transitivity measures the degree of clustering within a network and is defined as the number of completely connected triangles in a network divided by the number of triples – groups of three nodes – that are connected (Newman, 2003).

## Results

*Alpha diversity is weakly linked to watershed urbanization.* Relationships between bacterial diversity and habitat and watershed land cover type were detectable, but weak. Shannon Diversity was significantly higher for forested sites than urbanized sites ( $F_{(1,18)}=9.2$ ;  $p<0.01$ ) and higher in water column than sediment samples ( $F_{(1,18)}=6.39$ ;  $p<0.05$ ). Faith's Phylogenetic diversity was significantly higher in water column than sediment samples ( $F_{(1,18)}=16.9$ ;  $p<0.001$ ), but there was no significant difference in phylogenetic diversity between watershed land cover types. This trend was found with other measures as well, though differences for these metrics were not statistically significant (Figure 2).

Variability of alpha diversity over time was substantially lower for sediment samples than for water samples, especially for Shannon and Faith's Phylogenetic diversity. Diversity metrics were notably consistent for forested sediment samples where variation across samples was particularly low.

*Stream watershed land cover and sample habitat type are related to microbial beta diversity.* Microbial beta diversity showed distinct differences across the four combinations of stream watershed type and habitat. Taxa from the phylum Proteobacteria were a dominant taxonomic group in all sample types (Figure 3) with either Alphaproteobacteria or Gammaproteobacteria being the most abundant class in almost all samples. Urbanized water column samples generally had higher levels of Actinobacteria,

particularly in April 2012 and February 2013, and lower levels of Alphaproteobacteria and Acidobacteria compared to forested water column samples.

Principal coordinate analysis was conducted on Bray-Curtis distances between individual 16S rDNA samples showed that microbial community structure was strongly related to site (Figure 4a). The first principal coordinate axis differentiates between all four combinations of habitat and stream site type. Samples were grouped primarily accord to habitat; within each of these two clusters, samples from urbanized streams had higher principal coordinate 1 (PCo-1) scores than equivalent forested samples.

Both site watershed type ( $F_{(1,9)}=3.22$ ;  $p<0.01$ ) and habitat type ( $F_{(1,18)}=8.37$ ;  $p<0.01$ ) were significantly related to Bray-Curtis distances ( $r^2=0.38$ ) as determined by Adonis. To determine the relative influence of the two main effects on structuring of community composition similarity, ANOSIM was applied and a significant result ( $p<0.01$ ) was obtained. The global ANOSIM R statistic for all four combinations of habitat and watershed type was 0.74. The ANOSIM R for habitat was 0.78, greater than the global ANOSIM R, while the R statistic for watershed type (0.23) was the lowest value. Thus, both sediment cover and habitat type were significantly correlated to bacterial community composition, with habitat appearing to have a stronger effect than watershed type.

The OTUs with the greatest degree of loading, and therefore the largest influence on principal coordinate ordination, were identified (Table 3.2). Higher PCo-1 scores were associated with urbanized and water column samples while lower PCo-1 scores were

associated with sediment and forest bacterial communities. The three OTUs with the highest PCo-1 scores were OTU 4 (genus *Polynucleobacter*), OTU 1 (genus *Albidiferax*), and OTU2 (uncultured Methylococcales clone CABC2E06). Two OTUs, OTU 5 (genus *Crenothrix*) and OTU 10 (Order Rhizobiales), had substantially negative PCo-1 scores.

*Environmental controls on microbial community composition.* A significant habitat type interaction was found when ANCOVA was applied to compare sample PCo-1 with log-transformed watershed impervious cover ( $F_{(1,18)}=4.57$ ;  $p<0.05$ ). Both water column ( $F_{(1,9)}=25.81$ ;  $p<0.001$ ) and sediment ( $F_{(1,9)}=9.96$ ;  $p<0.05$ ) samples were significantly related to log impervious cover, though the strength and slope of this relationship differed between the two sample types (Figure 4b). Overall, water column PCo-1 scores were more responsive to changes in log impervious cover, suggesting a stronger relationship between these two variables in water column than sediment samples.

Multivariate analysis using BV-STEP indicated that bacterial community composition in sediment was more strongly related to measured environmental variables than water column communities (Table 3.3). In sediments, stream discharge, water column DOC concentrations, and percent sediment carbon content – all variables log-transformed – were significantly linked to community composition during warm and cold seasons. In the water column, only log-transformed temperature and dissolved organic matter quality, as measured by FI, were significantly linked to community composition across seasons. Variables positively correlated with urbanization including FI, TN, conductivity, temperature, and DON all covaried and were negatively related to sediment C:N,

sediment percent carbon, and water column DOC concentration, variables which are associated with forested sites (Figure 5).

*Differences in microbial community composition identified with indicator species analysis.* A total of 312 taxa were identified as indicators of water column, sediment, urbanized stream site, or forested bacterial communities. Most taxa identified as indicators, 176, were associated with urbanized sites. The five most abundant OTUs from each indicator group are presented in Table 3.4. The five indicator OTUs with the greatest sequence abundance across all samples were all indicators of urban stream sites (Figure 6). An OTU belonging to the genus *Polynucleobacter* (OTU 4), and an indicator of urbanized sites, was by far the most abundant indicator taxon with 26,374 sequences across all samples. The most abundant indicator OTU not associated with urbanized samples was the genus *Hyphomicrobium*, which was an indicator of samples from forested sites.

Both OTUs and samples were arranged on the heat map by cluster analysis using Bray-Curtis distances between samples. The heat map showed that while individual OTUs were not entirely diagnostic, the twenty OTUs identified as top indicators could be used to group sites according to the relevant indicator categories (Figure 3.6). Indicator OTUs were also almost entirely correctly grouped according to indicator category.

*Microbial co-occurrence networks change with landscape urbanization.* To compare co-occurrence relationships of microbial communities collected from stream samples, four

networks were generated (Figure 7) for each of the four combinations of site type (forested and urbanized) and habitat type (sediment and water column). The results indicated that network relationships did change significantly across the four networks ( $p < 0.001$ ,  $r^2 < 0.48$ ).

Fewer taxa were included in the forested networks compared to the urbanized networks; however, these networks displayed higher transitivity – a measure of connectedness within clusters – and lower modularity – an indicator of subgroup aggregation in a network – than urbanized networks (Table 3.5). This was particularly true of the forested sediment network, which had a very high average node degree – the number of edges connected to a node (Freeman, 1978) – and low modularity, indicating a high degree of co-occurrence links across the network. The network of forested water column samples had lower average node degree than the urbanized networks (13.1), but distribution of node degree across taxa was similar to the forested sediment network (Figure 8). The distribution of node degrees of forested networks, particularly the forested sediment network, was bimodal with some highly connected taxa at the center of the network and a larger group of taxa with relatively low node degree. In contrast, node degree distribution of the urbanized networks was log-normal. Altered distribution of node weights, combined with higher modularity and lower transitivity of these networks, indicate that urbanized microbial networks are comprised of several aggregates of taxa that are highly internally connected, but not well connected to other aggregates.

While the network structures were similar amongst forested and urbanized sites respectively, the taxa contributing to each network differed substantially. This was particularly true between forested and urbanized networks (Table 3.6). In forested microbial networks (Figure 8a and b), a large proportion of the most central taxa – as indicated by high node degree – are from Alphaproteobacteria and Betaproteobacteria. By contrast, in the urbanized networks a high proportion of the most central taxa were from the classes Cytophagia and Nitrospira.

To determine what microbial associations remained important across sample types, networks were compared for common edges. Edges represent significant co-occurrence relationships between two taxa. Across the four networks, the only common significant relationship between two OTUs was between an OTU 440 (family Erythrobacteraceae) and OTU 46371 (family Rhodobacteraceae OTU 46371). Forested, urbanized, and water column networks all contained a number of significant interactions that included OTU 1 (genus *Albidiferax*), but this taxon did not appear in the sediment network. By contrast, a number of the top edges in sediment samples included OTU 5 (genus *Crenothrix*), a methane-oxidizing gammaproteobacterium (Stoecker et al., 2006). In urbanized sites *Polynucleobacter* (OTU 4) showed significant co-occurrence patterns with several taxa.

## **Discussion**

*Stream watershed land cover and microbial habitat related to microbial community composition.* Microbial communities in Parkers Creek headwater streams appear to be structured by microbial habitat (sediment, water column) and stream land cover type

(forested, urbanized), supporting the hypothesis that these two factors drive microbial community structure in these systems. Statistical analyses and a PCoA biplot (Figure 4a), indicate that microbial community composition is structured primarily by habitat type with stream land cover type acting as a secondary factor.

While dispersal from the watershed controls which microbes are available to colonize streambed sediments (Crump et al. 2012), only a portion of the microbial community dispersed through the water column is able to develop in or on the more stable sediment habitat (Crump et al., 2007, 2007; Adams et al., 2014). Thus, local sediment conditions may be more responsible for determining which microbes can persist and become established. By contrast, watershed environmental conditions and land use should more directly influence bacterial community structure in the water column. In this study, I found evidence that Microbial community composition and watershed impervious cover were significantly related in both water and sediment habitats (Figure 3.4b), but with a weaker response found for sediment compared to water column bacterial community composition. Environmental conditions and bacterial community composition were more strongly linked in sediments than water column, supporting the assertion that streambed sediment communities are filtered by conditions at a site.

*Community composition changes with environment.* Community composition, as measured by Bray-Curtis distances, demonstrated a continual gradient across samples types from forested sediment communities to water column urbanized communities. The



result is that urbanized sediment communities are more similar to water column communities than are forested sediment communities.

Different methanotrophic taxa were identified as top indicators in both forested and urbanized streams. In urbanized streams, an OTU from the order Methylococcales, which is comprised exclusively of methanotrophs (Bowman, 2005; Kato et al., 2013), was a top indicator in urbanized streams. An OTU assigned to *Hyphomicrobium*, a facultative methylotroph (Scheuderman-Suylen and Kuenen, 1985), was a top indicator OTU in forested streams. Thus, while the dominant taxa were different between forested and urbanized sites, the functional capabilities in these two settings may be similar, which suggests a degree of functional redundancy across environments.

There are suggestions of a diverse community of denitrifying microbes in forested communities. *Hyphomicrobium* and *Rhizobiales* both include taxa capable of denitrification. *Nitrospira*, also a taxonomic group associated with forested samples, oxidize nitrite and is believed to support the functioning of anaerobic ammonia oxidizing (anammox) bacteria (Daims et al., 2001; Park et al., 2015).

Urbanized stream samples include taxa that are indicative of eutrophication and urban impacts. *Polynucleobacter*, abundant in urbanized stream samples, is a picobacter often associated with high levels of planktonic autotrophic activity and warm temperatures (Hahn, 2003; Boenigk et al., 2004; Wu and Hahn, 2006). The presence of *Polynucleobacter* is thus an indicator of the establishment of a more planktonic microbial

community, a sign of eutrophic conditions in urbanized streams. *Albidiferax* species have been found ubiquitously in samples of groundwater contaminated with tetrachlorethene (Kotik et al., 2013). *Gallionella*, also more abundant in urbanized Parkers Creek streams, is responsible for corrosion in water distribution systems (Ridgway et al., 1981). Thus, all of these taxa can be associated with increased impact of human systems.

*Environmental controls on bacterial diversity.* The primary environmental factors related to bacterial community structure were all related to watershed urbanization. Variables identified in this study such as carbon quality (Crump et al., 2003; Judd et al., 2006; Besemer et al., 2013) and quantity (Beier et al., 2008; Larouche et al., 2012), nitrogen and phosphorus levels (Makino and Cotner, 2004; Larouche et al., 2012), and water conductivity (Beier et al., 2008; Zeglin et al., 2011) and pH (Fierer et al., 2007), have all been previously related to community structure in aquatic systems. Thus, the results reported here suggest that urbanization structures microbial communities via the same mechanisms previously identified in less anthropogenically impacted systems.

Interestingly, discharge was a significant factor for models of sediment communities, but not water column communities. This may be due to the influence of flow conditions on biofilm and community structure of streambed microbes (Besemer et al., 2009a, 2009b). Discharge may also indirectly impact microbial community structure by altering bed sediment size (Jackson and Weeks, 2008; Perryman et al., 2011b).

Many of the factors related to community structure in these streams, including carbon quality, nutrient ratios, and conductivity were shown in Chapter 1 to be correlated to impervious cover. Thus, further work with controlled experiments linking community structure to particular environmental changes will be required to uncover mechanistic controls on bacterial communities.

*Microbial networks change with urbanization.* The structure of microbial networks differed significantly across environments, partially supporting the third hypothesis that key microbial interactions would differ between site and habitat type. It was predicted that microbial network structure would change in response to both stream watershed type and habitat type; however, changes to microbial co-occurrence networks were more strongly linked to degree of urbanization rather than habitat (Figures 3.7 and 3.8). This stands in contrast to microbial community composition in streambed sediments, which was more strongly linked to site-specific habitat conditions. Clearly analysis of network and community structure captures different aspects of microbial diversity; however, the implications for microbial function of these differing metrics remains substantially less clear.

In contrast to my hypothesis, urbanized networks demonstrated greater modularity and lower average path length and transitivity than equivalent forested networks, indicating less connectivity among subgroups within those networks (Faust et al., 2012). Microbial networks with higher modularity are found in more disturbed ecosystems (Parter et al.,

2007), suggesting that the increased disturbance regime of urbanized streams is having a fundamental impact on stream microbial interactions.

In forested stream networks, one group of keystone taxa with high node degree lies at the center of the network. In urbanized networks, the network has been divided into relatively independent aggregates, as indicated by the higher modularity (Table 3.7): networks with values above 0.4 are considered modular (Newman, 2003). The result is a shift in the way microbial co-occurrence is structured (Figure 8). Instead of a few strong keystone taxa, as is the case in forested networks, urbanized streams have a larger number of weaker keystone taxa spread across disparate portions of the more modular urbanized networks. Thus, structure of microbial interactions in urbanized streams appear to be fundamentally different than forested streams, yet how this change impacts the stability and functional activity of microbial communities is unclear.

Common edges between networks represent co-occurrence events between two OTUs that are observed across all networks being compared. These common edges can be applied to assess what microbial associations are shared across different environments (Williams et al., 2014) and therefore what taxa remain important across networks from differing environments. Only one microbial association, between taxa from *Erythrobacteraceae* (OTU 440) and *Rhodobacteraceae* (OTU 46371), was found in all four microbial networks. The family *Erythrobacteraceae* are heterotrophs that are found across a wide range of environments (Tonon et al., 2014). Members of the family *Rhodobacteraceae* are important as the first members of biofilms formed in

Mediterranean seawater (Elifantz et al., 2013). This evidence suggests the interaction between these two taxa is important for biofilm formation in freshwater streams as well as seawater.

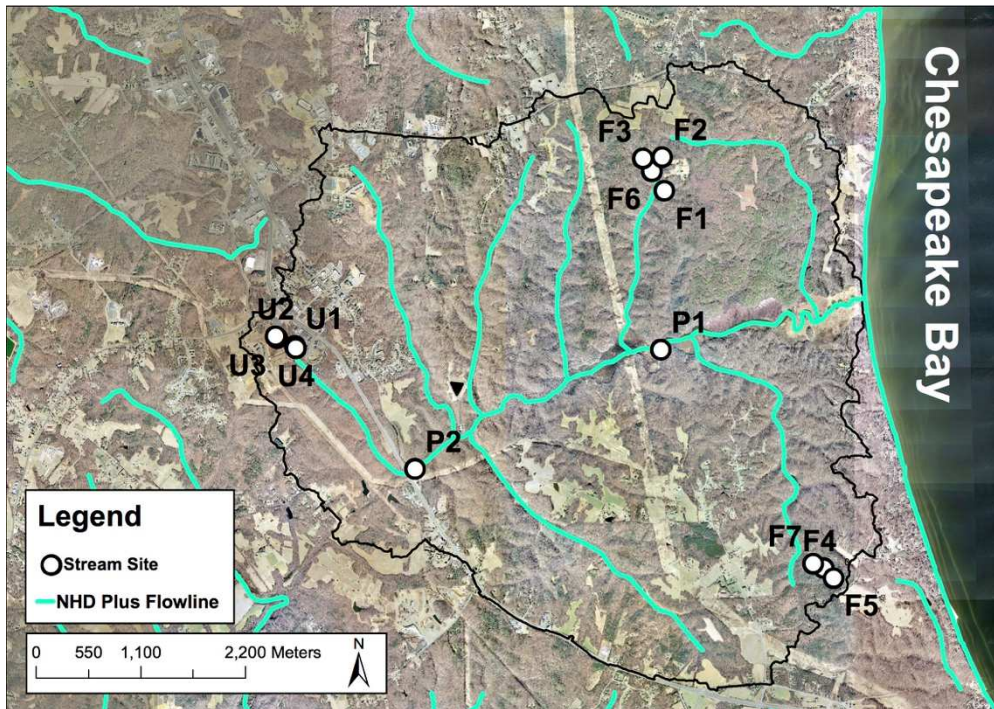
Water column, forested, and urbanized networks all showed an abundance of links between *Albidiferax* (OTU 1) and other OTUs. *Albidiferax ferrireducens*, the only member of the genus, reduces iron as a means of respiration and has been found to associate with iron-rich resources in a variety of environments, including lake and marine settings (Lu et al., 2013a; Elser et al., 2015). Co-occurrence events between *Albidiferax* and an OTU from the order Desulfuromonadales, another iron reducing taxon (Lovley, 2006; Prakash et al., 2010), were found in urbanized networks (Table 3.8). Thus, plentiful network associations with *Albidiferax* may be indicative of communities adapted to anaerobic respiration.

*Conclusions.* Differences in microbial community composition across forested and urbanized headwater streams were most strongly related to habitat (water vs. sediment). Differences between bacterial communities in samples from urbanized and forested stream sites were also found, but this change was a secondary factor to habitat type. Bacterial community structure showed little change over the course of the study, with community composition at each site remaining relatively consistent. Together, these lines of evidence indicate a strong role for environmental sorting.

Sediment and water column communities shifted similarly with response to urbanization. This shift suggests that a change in the community composition in the water column exerted an impact on urbanized sediment microbial community structure. Such an influence suggests a secondary role for dispersal in determining microbial community in sediments. This evidence is not definitive; however, considering that changes in the sediment environment caused by urbanization may have changed microbial niches resulting in different taxonomic composition, as has been seen for denitrifier communities (Perryman et al., 2011b).

Changes to microbial associations, as measured by microbial co-occurrence networks, indicate that microbial assemblages change substantially in response to urbanization. Urbanized networks were more modular, whereas forested networks contained a few keystone taxa that were central to the entire network. Changes in community relationships between forested and urbanized networks indicate shifting patterns of microbial interaction that may result in changes to microbial function. Future studies should combine biogeochemical and molecular approaches to determine the functional significance of shifting microbial networks.

## Tables and Figures

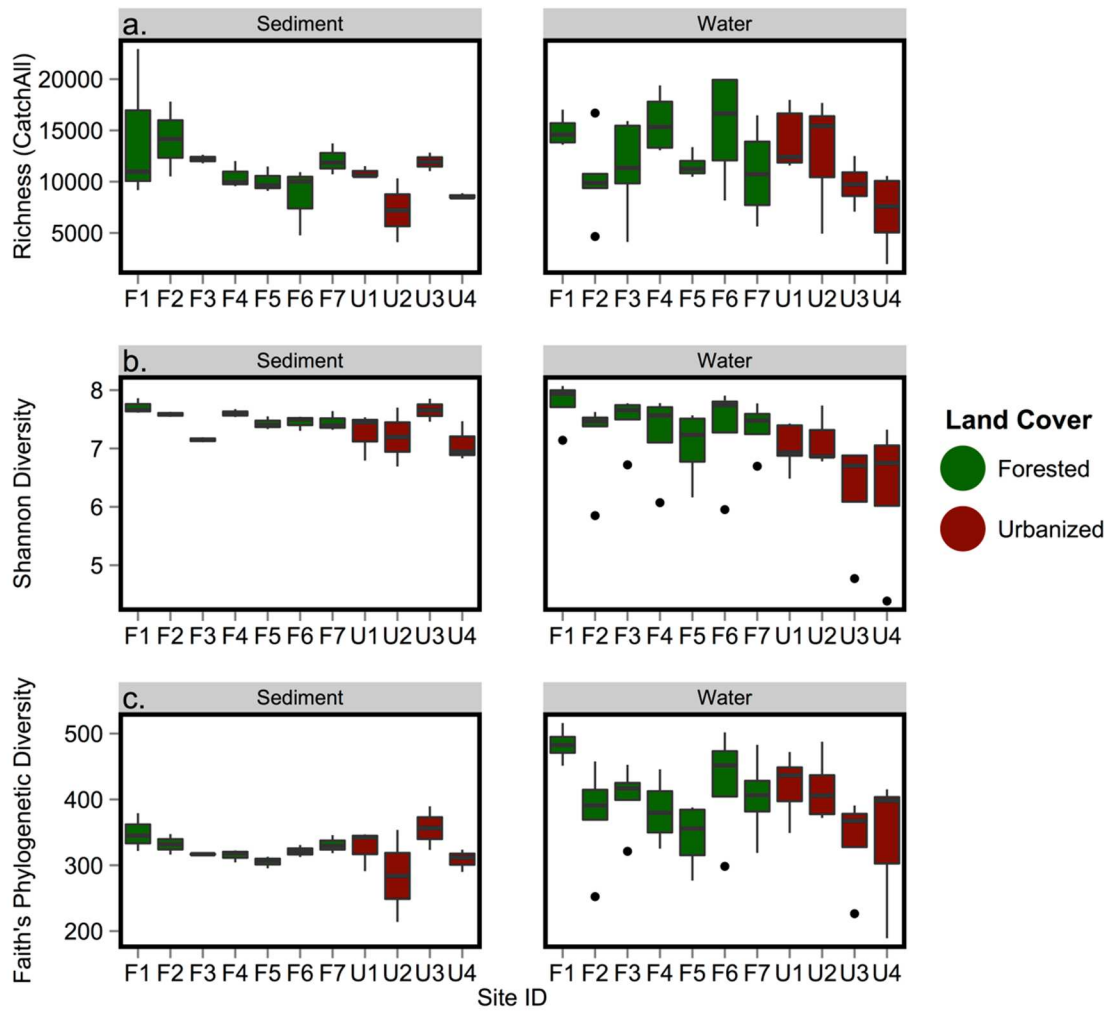


**Figure 3.1:** A map of the Parkers Creek watershed with the study sites indicated. For first order sites for which there is more than one sampling site (e.g. F1, F1-A, F1-B), only one site is indicated. Some stream sampling sites are located in channels that are not included in NHD Plus maps. Aerial orthophotography was provided by Calvert County, Maryland government.

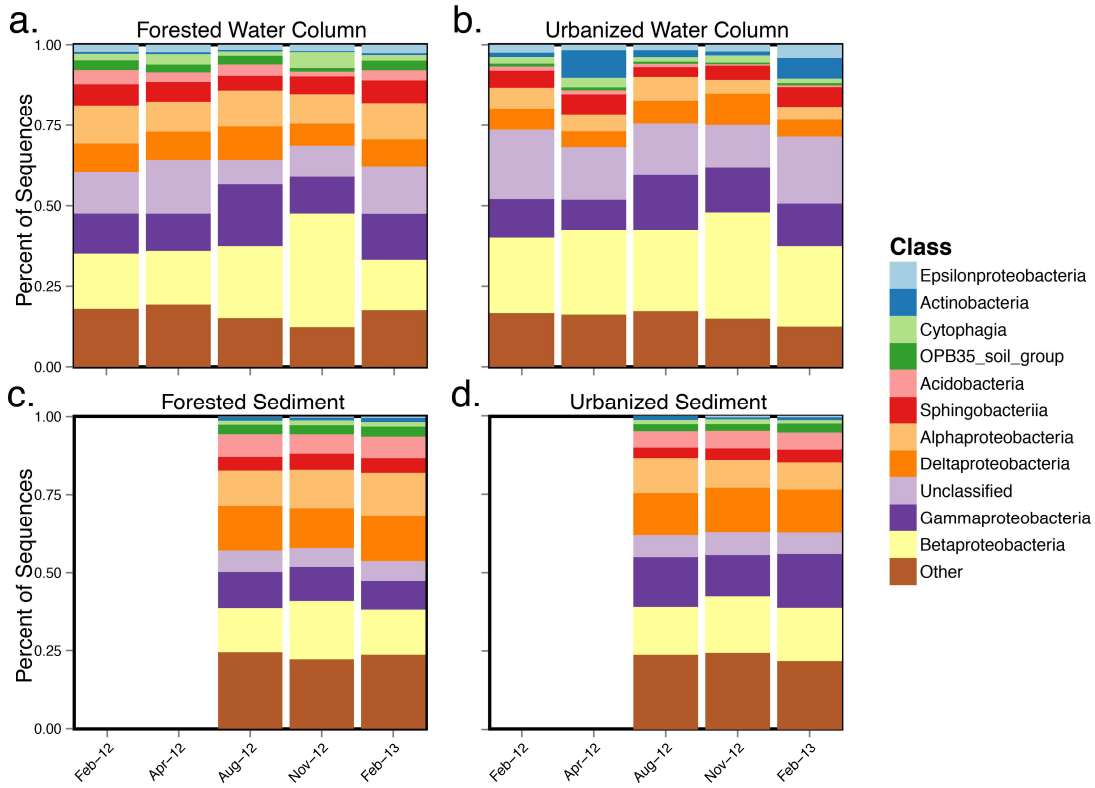
**Table 3.1:** Summary of site identities, watershed area, and location.

<b>Site</b>	<b>Primary Land Cover</b>	<b>Stream Reach Order</b>	<b>Watershed Area (Hectares)</b>	<b>Latitude</b>	<b>Longitude</b>	<b>% Forest Cover</b>	<b>% Impervious Cover</b>
<b>F1</b>	Forested	First	11.70	38°32'51.31"N	76°32'28.97"W	90.6%	0.4%
<b>F2</b>	Forested	First	4.413	38°33'01.94"N	76°32'30.23"W	64.4%	4.9%
<b>F3</b>	Forested	First	6.970	38°33'01.62"N	76°32'39.14"W	94.7%	2.1%
<b>F4</b>	Forested	First	2.855	38°30'41.87"N	76°31'21.21"W	100%	0.0%
<b>F5</b>	Forested	First	2.778	38°30'38.71"N	76°31'16.67"W	90.8%	2.0%
<b>U1</b>	Urbanized	First	1.530	38°31'58.57"N	76°35'09.51"W	33.5%	44.0%
<b>U2</b>	Urbanized	First	7.708	38°32'01.84"N	76°35'18.19"W	27.7%	24.3%
<b>U3</b>	Urbanized	First	4.693	38°32'00.43"N	76°35'17.90"W	2.5%	10.0%
<b>F7</b>	Forested	Second	16.51	38°30'44.08"N	76°31'25.32"W	93.0%	2.5%
<b>F6</b>	Forested	Second	21.38	38°32'57.27"N	76°32'35.09"W	86.5%	1.7%
<b>U4</b>	Urbanized	Second	27.51	38°31'57.68"N	76°35'09.02"W	24.2%	24.1%

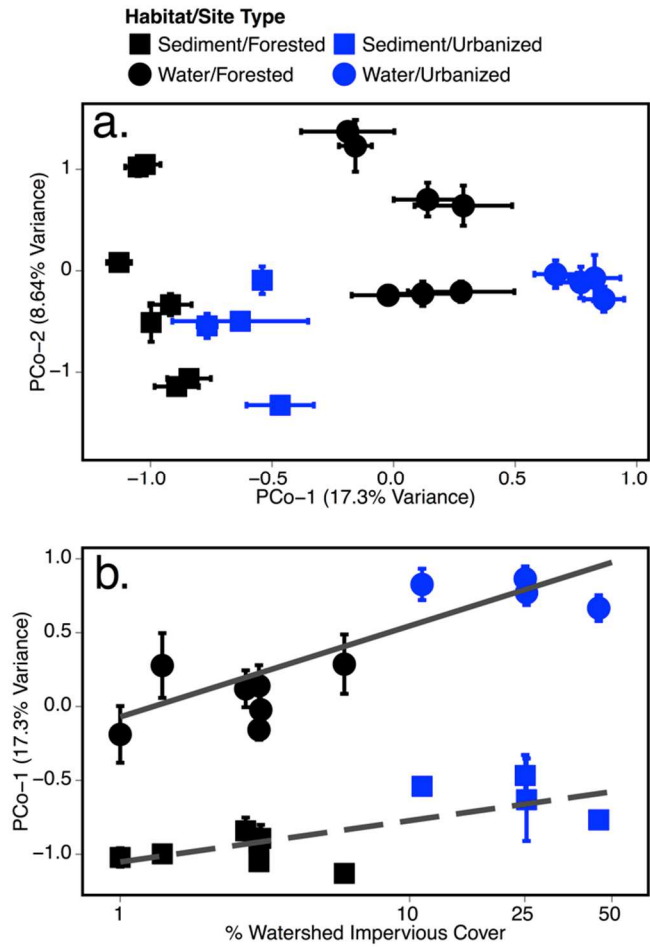




**Figure 3.2:** Boxplots of (a) OTU richness, as measured by CatchAll, (b) Shannon Diversity, and (c) Faith's Phylogenetic Diversity of microbial OTUs as developed from 16S rDNA analysis. Boxplots represent variation over repeated collections of sediment and water samples respectively from February 2012 through February 2013.



**Figure 3.3:** Mean class-level composition over time for (a) forested water column, (b) urbanized water column, (c) forested sediment, and (d) urbanized sediment samples.



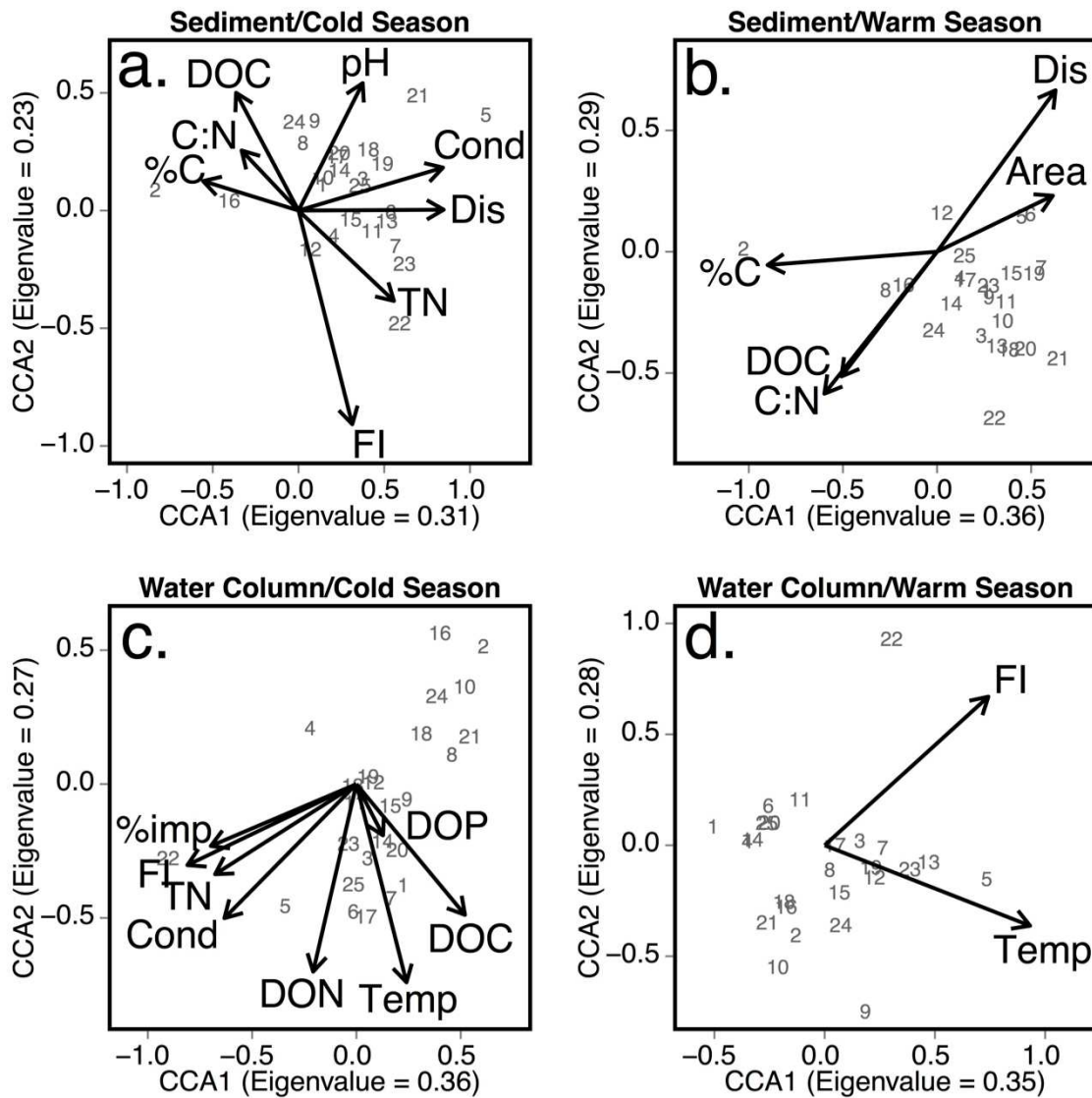
**Figure 3.4:** Biplot of (a) the first two PCoA scores of microbial OTU Bray-Curtis distances plotted by site and habitat type (sediment and water column) and (b) percent watershed impervious cover at a site versus principal coordinate axis 1 (PCo-1) scores. Each point is identified according to site land cover type (forested and urbanized) and sample habitat (sediment and water column). Error bars represent standard error from repeated measurements taken February 2012-February 2013.

**Table 3.2:** OTUs with highest total loadings for principal coordinate (PCo) dimensions 1 and 2.

<b>OTU ID</b>	<b>PCo-1 Score</b>	<b>PCo-2 Score</b>	<b>OTU Taxonomy</b>
OTU 1	1.213	0.554	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Albidiferax
OTU 2	0.801	-0.625	Bacteria Proteobacteria Gammaproteobacteria Methylococcales CABC2E06 NC
OTU 4	1.384	-0.194	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Burkholderiaceae Polynucleobacter
OTU 5	-0.496	-0.674	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
OTU 10	-0.376	0.393	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales NC
OTU 12	0.496	0.438	Bacteria Proteobacteria Epsilonproteobacteria Campylobacterales Helicobacteraceae Sulfuricurvum
OTU 13	0.148	0.759	Bacteria Proteobacteria Gammaproteobacteria Legionellales Coxiellaceae Rickettsiella
OTU 14	0.657	-0.170	Bacteria Proteobacteria Betaproteobacteria Nitrosomonadales Gallionellaceae Gallionella
OTU 18	0.409	-0.107	Bacteria Actinobacteria Actinobacteria Frankiales Sporichthyaceae Candidatus Planktophilia
OTU 19	0.427	-0.063	Bacteria Bacteroidetes Cytophagia Cytophagales Cytophagaceae Arcicella

**Table 3.3:** Variables related to microbial community structure using the BV-STEP function. Model fit is reported as BV-STEP  $\rho$  and canonical correspondence analysis  $R^2$ .

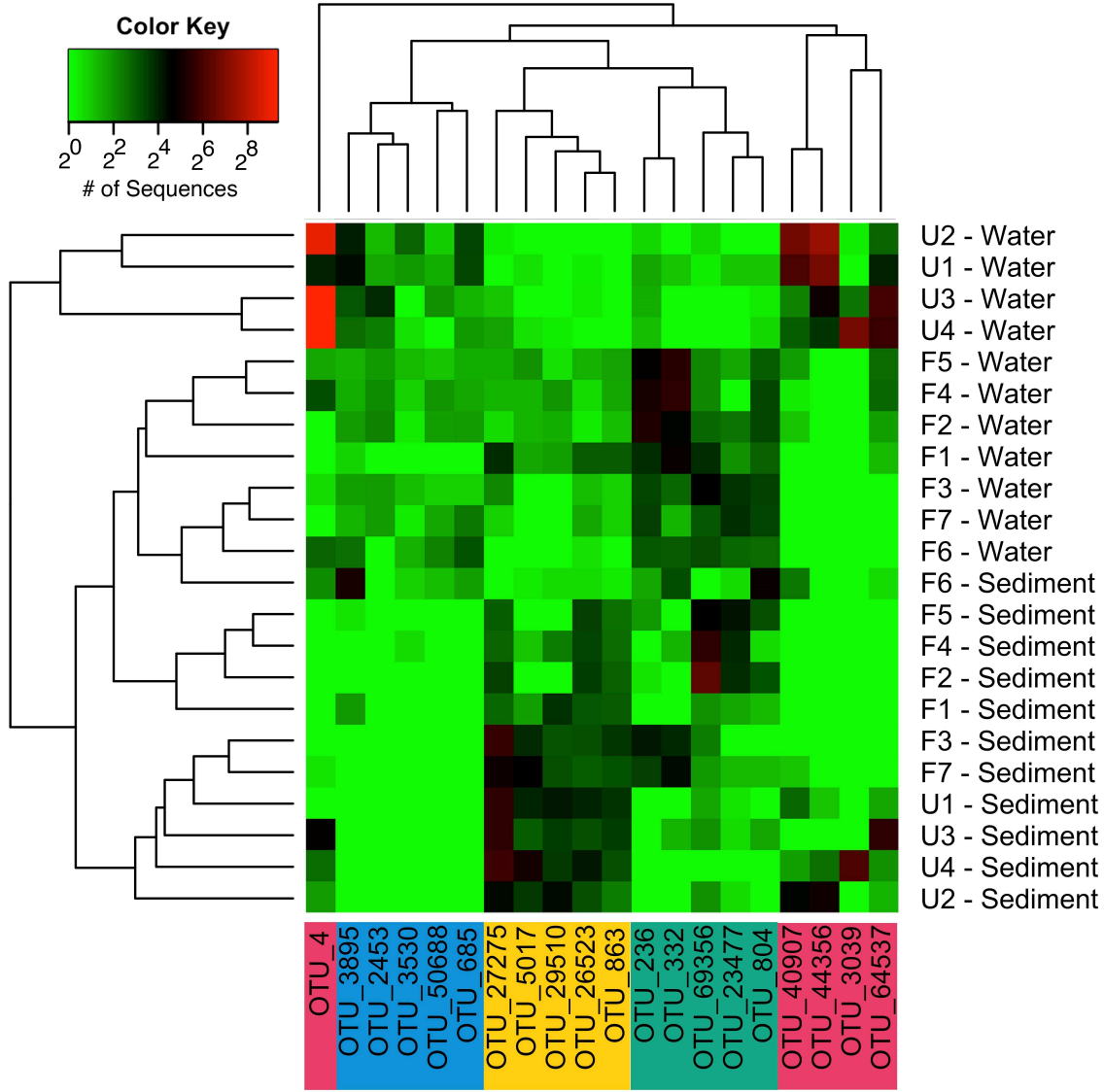
<b>Sample Type</b>	<b>Significant variables</b>	<b><math>\rho</math> (BV-STEP)</b>	<b><math>R^2</math> (CCA)</b>
Cold Season/ Sediment	log(Discharge), log(DOC), log(TN), log(FI), log(Conductivity), log(Sediment %C), log(Sediment C:N), pH	0.705	0.544
Warm Season/ Sediment	log(Discharge), log(DOC), log(Sediment %C), log(Sediment C:N), log(Watershed Area)	0.680	0.783
Cold Season/ Water Column	log(DOC), log(TN), log(FI), log(Conductivity), log(Ortho-Phosphate), log(DON), log(% Impervious Cover), log(Temperature)	0.558	0.348
Warm Season/ Water Column	log(FI), log(Temperature)	0.376	0.169



**Figure 3.5:** Biplots of canonical correspondence analysis of environmental factors (indicated by arrows) and top OTUs (identified by number) in a) sediment during cold seasons, b) sediment during warm seasons, c) water column during cold seasons, and d) water column during warm seasons. Taxonomic identification of the OTUs included in the plots is included in Table 3.4.

**Table 3.4:** Taxonomic identification of OTUs included in plots presented in Figure 3.5.

Plot ID	OTU ID	Taxonomic Classification
1	OTU_1	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Albidiferax
2	OTU_10	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae Unclassified
3	OTU_11	Bacteria Proteobacteria Betaproteobacteria Methylophilales Methylophilaceae Unclassified
4	OTU_12	Bacteria Proteobacteria Epsilonproteobacteria Campylobacterales Helicobacteraceae Sulfuricum
5	OTU_2	Bacteria Proteobacteria Gammaproteobacteria Methylococcales CABC2E06 Unclassified
6	OTU_27234	Bacteria Proteobacteria Deltaproteobacteria Desulfuromonadales M20-Pitesti Unclassified
7	OTU_28	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Unclassified
8	OTU_31	Bacteria Proteobacteria Alphaproteobacteria Sphingomonadales Sphingomonadaceae Novosphingobium
9	OTU_32248	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Unclassified
10	OTU_34	Bacteria Proteobacteria Betaproteobacteria Unclassified Unclassified Unclassified
11	OTU_35775	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
12	OTU_41	Bacteria Acidobacteria Holophagae Holophagales Holophagaceae Geothrix
13	OTU_5	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
14	OTU_54	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Rhizobacter
15	OTU_55365	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Unclassified
16	OTU_56	Bacteria Nitrospirae Nitrospira Nitrospirales 4-29 Unclassified
17	OTU_63	Bacteria Proteobacteria Deltaproteobacteria Desulfuromonadales BVA18 Unclassified
18	OTU_70205	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Unclassified Unclassified
19	OTU_70675	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Unclassified
20	OTU_71884	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Unclassified
21	OTU_75	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Unclassified Unclassified
22	OTU_78	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
23	OTU_8	Bacteria Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
24	OTU_87	Bacteria Proteobacteria Gammaproteobacteria Xanthomonadales Sinobacteraceae Unclassified
25	OTU_9	Bacteria Proteobacteria Deltaproteobacteria Desulfuromonadales BVA18 Unclassified

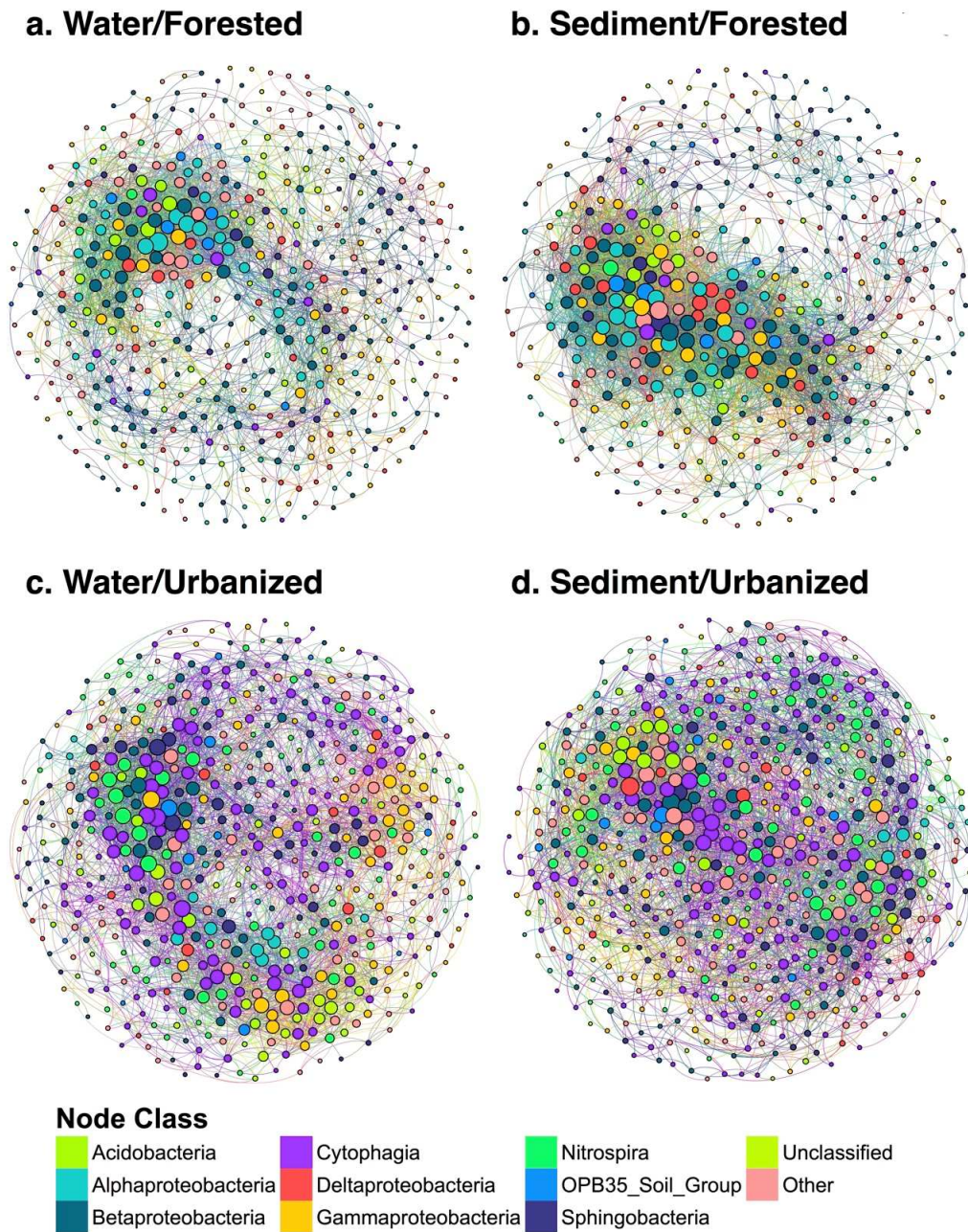


**Figure 3.6:** A heatmap of most abundant indicator species for samples from water column, sediment, urbanized sites, and forested sites. Cluster analysis was performed using Bray-Curtis distances. Cell color indicates mean sequence abundance over the course of the study of OTUs by site and habitat type; note that color is scaled logarithmically. OTUs are color-code according to indicator group: pink = urbanized, green = forested, blue = water column, and yellow = sediment).



**Table 3.5:** The most abundant taxa from each of the four indicator groups as identified by indicator species analysis.

OTU ID	Indicator Cluster	P-Value	Indicator Statistic Value	Total Number of Sequences	Taxonomy
OTU 3039	Urbanized	0.005	0.445	1191	Bacteria Proteobacteria Gammaproteobacteria Methylococcales NC NC
OTU 64537	Urbanized	0.005	0.868	1215	Bacteria Bacteroidetes Sphingobacteriia Sphingobacteriales Chitinophagaceae NC
OTU 40907	Urbanized	0.010	0.826	1283	Bacteria Proteobacteria Betaproteobacteria Nitrosomonadales Gallionellaceae Gallionella
OTU 44356	Urbanized	0.005	0.654	2078	Bacteria Proteobacteria Betaproteobacteria NC NC NC
OTU 4	Urbanized	0.005	0.892	26374	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Burkholderiaceae Polynucleobacter
OTU 29510	Sediment	0.050	0.701	349	Bacteria Bacteroidetes vadinHA17 NC NC NC
OTU 863	Sediment	0.050	0.878	389	Bacteria Proteobacteria Deltaproteobacteria Myxococcales Haliangiaceae Haliangium
OTU 5017	Sediment	0.020	0.814	417	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales MNG7 NC
OTU 26523	Sediment	0.010	0.887	487	Bacteria Acidobacteria Acidobacteria DA023 NC NC
OTU 27275	Sediment	0.035	0.939	893	Bacteria Nitrospirae Nitrospira Nitrospirales Nitrospiraceae Nitrospira
OTU 69356	Forested	0.005	0.835	575	Bacteria Proteobacteria Gammaproteobacteria NC NC NC
OTU 23477	Forested	0.010	0.731	610	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales NC NC
OTU 236	Forested	0.005	0.757	812	Bacteria Bacteroidetes Sphingobacteriia Sphingobacteriales Chitinophagaceae Hydrotalea
OTU 332	Forested	0.005	0.789	835	Bacteria Bacteroidetes NC NC NC NC
OTU 164	Forested	0.050	0.800	1006	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Hyphomicrobiaceae Hyphomicrobium
OTU 1364	Water	0.045	0.730	171	Bacteria Candidate division OP3 NC NC NC NC
OTU 2212	Water	0.050	0.815	223	Archaea Euryarchaeota Halobacteria Halobacteriales DHVEG-6 Candidatus Parvarchaeum
OTU 50688	Water	0.035	0.631	314	Bacteria Proteobacteria Betaproteobacteria Burkholderiales Oxalobacteraceae NC
OTU 685	Water	0.020	0.856	333	Bacteria Candidate division OD1 NC NC NC NC
OTU 10693	Water	0.050	0.867	619	Bacteria Candidate division OP3 NC NC NC NC



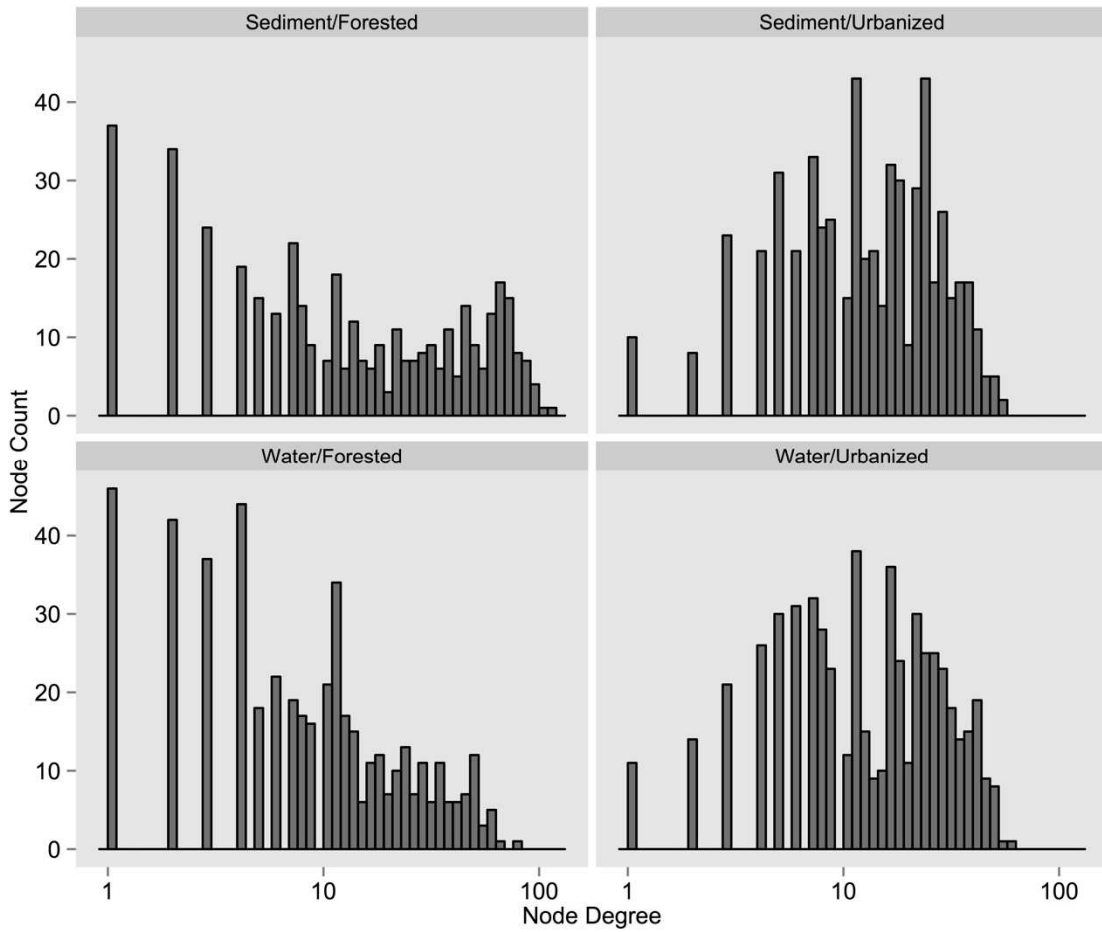
**Figure 3.7:** Microbial co-occurrence networks from a) water column forested, b) sediment forested, c) water column urbanized, and d) sediment urbanized samples. Each node represents an individual taxon. Each node is color coded according to its taxonomic class and the size of the node is degree, which is the number of vertices that connect to that node.

**Table 3.6:** Table of the OTUs with the highest node degree in each of the four networks analyzed.

OTU ID	Network	Degree	Between-ness	Close-ness	Taxonomy
OTU 127	Sediment/Forested	94	0.0202	0.391	Bacteria Verrucomicrobia OPB35 soil_group Unclassified Unclassified Unclassified
OTU 159	Sediment/Forested	97	0.0124	0.397	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae Rhodoblastus
OTU 8474	Sediment/Forested	97	0.0113	0.396	Bacteria Proteobacteria Betaproteobacteria Unclassified Unclassified Unclassified
OTU 488	Sediment/Forested	103	0.0132	0.401	Bacteria Bacteroidetes SB-1 Unclassified Unclassified Unclassified
OTU 148	Sediment/Forested	112	0.0273	0.412	Bacteria Bacteroidetes Sphingobacteriia Sphingobacteriales Chitinophagaceae Unclassified
OTU 8474	Sediment/Urbanized	49	0.0100	0.368	Bacteria Proteobacteria Betaproteobacteria Unclassified Unclassified Unclassified
OTU 348	Sediment/Urbanized	50	0.0062	0.362	Bacteria Nitrospirae Nitrospira Nitrospirales 0319-6A21 Unclassified
OTU 473	Sediment/Urbanized	50	0.0068	0.363	Bacteria Planctomycetes Planctomycetacia Planctomycetales Planctomycetaceae Planctomyces
OTU 17376	Sediment/Urbanized	55	0.0093	0.366	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Xanthobacteraceae Unclassified
OTU 287	Sediment/Urbanized	56	0.0261	0.371	Bacteria Bacteroidetes Cytophagia Cytophagales Unclassified Unclassified
OTU 74	Water/Forested	60	0.0064	0.327	Bacteria Proteobacteria Gammaproteobacteria Xanthomonadales Sinobacteraceae Unclassified
OTU 159	Water/Forested	63	0.0093	0.335	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae Rhodoblastus
OTU 164	Water/Forested	63	0.0174	0.343	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Hyphomicrobiaceae Hyphomicrobium
OTU 51	Water/Forested	67	0.0096	0.341	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales alphaI cluster Unclassified
OTU 10	Water/Forested	79	0.0184	0.342	Bacteria Proteobacteria Alphaproteobacteria Rhizobiales Bradyrhizobiaceae Unclassified
OTU 36512	Water/Urbanized	52	0.0132	0.357	Bacteria Nitrospirae Nitrospira Nitrospirales Nitrospiraceae Nitrospira
OTU 48993	Water/Urbanized	52	0.0057	0.353	Bacteria Proteobacteria Betaproteobacteria Unclassified Unclassified Unclassified
OTU 6130	Water/Urbanized	52	0.0193	0.368	Bacteria Proteobacteria Betaproteobacteria Rhodocyclales Rhodocyclaceae Sulfuritalea
OTU 320	Water/Urbanized	55	0.0054	0.352	Bacteria Proteobacteria Deltaproteobacteria Incertae Sedis Syntrophorhabdaceae Syntrophorhabdus
OTU 44758	Water/Urbanized	59	0.0108	0.359	Bacteria Proteobacteria Betaproteobacteria Nitrosomonadales Gallionellaceae Unclassified

**Table 3.7:** Network statistics of the microbial co-occurrence networks. Transitivity and modularity are normalized.

<b>Matrix</b>	<b>Watershed Land Use</b>	<b>Average Path Length</b>	<b>Transitivity</b>	<b>Modularity</b>	<b>Average Node Degree</b>	<b># Nodes</b>	<b># Edges</b>
Water	Forested	3.957	0.453	0.445	13.1	483	3162
Sediment	Forested	3.501	0.531	0.334	23.9	414	4956
Water	Urbanized	3.437	0.432	0.526	16.9	559	4711
Sediment	Urbanized	3.348	0.399	0.501	16.6	567	4694



**Figure 3.8:** Histograms of node degree for each of the four networks generated. Note that x-axes are log-transformed.

**Table 3.8:** Edges shared in common between two or more different networks. Edges are significant relationships between a pair of OTUs as indicated by Spearman's Rank Correlation. The top five edges from each network comparison are shown. Comparisons applied: sediment (forested/sediment & urbanized/sediment), forested (forested/sediment & forested/water column), urbanized (Urbanized/Sediment & Urbanized/Water Column), water column (forested/water column & urbanized/water column), and all networks. The number of OTU pairs shared between networks of a particular type is given by the last column. Only one edge was shared between all four networks. Taxonomic identity of OTUs is described in Table 6.

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Networks	OTU #1	OTU #2	No. of	No. of	No. Edges Shared
			Sequences: OTU #1	Sequences: OTU #2	
Sediment	OTU 30163	OTU 5	642	17810	328
Sediment	OTU 35471	OTU 5	866	17810	
Sediment	OTU 1066	OTU 5	1028	17810	
Sediment	OTU 17376	OTU 8	1301	19187	
Sediment	OTU 2	OTU 47067	26647	1323	
Forested	OTU 1	OTU 55	60515	1719	614
Forested	OTU 1	OTU 26	60515	4385	
Forested	OTU 1	OTU 32694	60515	5462	
Forested	OTU 1	OTU 1898	60515	9017	
Forested	OTU 1	OTU 6	60515	11229	
Urbanized	OTU 113	OTU 4	2630	26374	191
Urbanized	OTU 22	OTU 4	6241	26374	
Urbanized	OTU 1898	OTU 4	9017	26374	
Urbanized	OTU 1	OTU 244	60515	1268	
Urbanized	OTU 1	OTU 9	60515	11350	
Water Column	OTU 41044	OTU 5	4614	17810	130
Water Column	OTU 1	OTU 58735	60515	774	
Water Column	OTU 1	OTU 66564	60515	1093	
Water Column	OTU 1	OTU 26	60515	4385	
Water Column	OTU 1	OTU 32694	60515	5462	
All Networks	OTU 440	OTU 46371	693	519	1

**Table 3.9:** Taxonomy of OTUs included in Table 3.8.

<b>OTU ID</b>	<b>OTU Taxonomy</b>
OTU 1	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Albidiferax
OTU 2	Proteobacteria Gammaproteobacteria Methylococcales CABC2E06 NC
OTU 4	Proteobacteria Betaproteobacteria Burkholderiales Burkholderiaceae Polynucleobacter
OTU 5	Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
OTU 6	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Polaromonas
OTU 8	Proteobacteria Gammaproteobacteria Methylococcales Crenotrichaceae Crenothrix
OTU 9	Proteobacteria Deltaproteobacteria Desulfuromonadales BVA18 NC
OTU 22	Actinobacteria Actinobacteria Frankiales Sporichthyaceae hgcI clade
OTU 26	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae Rhodobacter
OTU 55	Bacteroidetes Sphingobacteriia Sphingobacteriales Chitinophagaceae Hydrotalea
OTU 113	Proteobacteria Deltaproteobacteria Myxococcales Cystobacteraceae Anaeromyxobacter
OTU 244	Bacteroidetes Sphingobacteriia Sphingobacteriales Chitinophagaceae NC
OTU 440	Proteobacteria Alphaproteobacteria Sphingomonadales Erythrobacteraceae NC
OTU 1066	Proteobacteria Gammaproteobacteria Methylococcales Methylococcaceae Methylosoma
OTU 1898	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae NC
OTU 17376	Proteobacteria Alphaproteobacteria Rhizobiales Xanthobacteraceae NC
OTU 30163	Verrucomicrobia Opitutae Opitales Opitutaceae Opitutus
OTU 32694	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae NC
OTU 35471	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Limnohabitans
OTU 41044	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae NC
OTU 46371	Proteobacteria Alphaproteobacteria Rhodobacterales Rhodobacteraceae NC
OTU 47067	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae Limnohabitans
OTU 58735	Proteobacteria Betaproteobacteria Burkholderiales Comamonadaceae NC
OTU 66564	Proteobacteria Betaproteobacteria Rhodocyclales Rhodocyclaceae NC



## Summary and Conclusions

In this dissertation, I report on the relationship between stream microbial communities, organic carbon, and urbanization. To accomplish this, I first studied the quantity and quality of organic matter in headwater streams in response to urbanization and microbial metabolism of these resources [Chapter 1]. To quantify how microbial metabolic activity varies across time and space in headwater streams, I then applied enzyme activity studies [Chapter 2]. Finally, I examined the relationship between microbial community structure, habitat, and watershed urbanization [Chapter 3].

In Chapter 1, I significantly linked organic matter characteristics to catchment urbanization. The prevalence of humic DOM was negatively related to watershed impervious cover, while microbially produced fulvic acids became more dominant. Previous research has identified changes to organic matter due to point urban impacts like wastewater treatment plant effluent (Stedmon et al., 2003; Baker and Spencer, 2004; Sickman et al., 2007; Gücker et al., 2011). In this chapter, I report on a significant link identified between carbon quality and impervious cover in the absence of point sources. I also report in Chapter 1 on a positive correlation between microbial activity and carbon biodegradation. These findings demonstrate that urbanization shifts DOM quality, but also indicate that this alone is not sufficient to increase metabolism of DOM. Instead, the fate of carbon in freshwater ecosystems is tied to both carbon quality and microbial metabolic function.

In Chapter 2, I identified the environmental factors controlling microbial metabolism using extracellular enzyme activity (EEA) studies. I examined the relationship between microbial EEA and numerous variables related to stream physicochemical conditions and watershed characteristics. Unlike DOM quality, microbial EEA was generally not related to watershed urbanization. Of the seven enzymes assayed, only alkaline phosphatase activities were significantly related to watershed impervious cover in streams. Instead, temperature was most strongly linked to EEA, but the two variables demonstrated a nonlinear relationship. During cooler months, EEA was positively related to temperature. In warmer months, EEA was negatively related or unrelated to temperature. The result is that EEA was highest at moderate temperatures, implying that microbial communities in these streams are adapted to function at maximum rates under these conditions.

In Chapter 3, I report on the drivers of stream bacterial community composition and co-occurrence networks in Parkers Creek headwater streams. As in Chapter 1, I found a significant relationship between microbial community composition and urbanization. This difference was not as large, however, as the difference in microbial community composition between sediment and water column habitats. Microbial community structure was more strongly related to environmental variables in sediment than water column communities. I conclude from this evidence that established bacterial communities in sediments are more strongly shaped by environmental conditions than the more ephemeral communities found in the water column. In contrast to overall community structure, microbial co-occurrence networks were more strongly linked to watershed type than habitat type. Microbial networks from urbanized streams showed

higher redundancy and modularity than forested streams. High modularity in bacterial communities has been experimentally linked to higher levels of disturbance (Newman, 2006; Parter et al., 2007; Olesen et al., 2007), indicating that impacts from urban landscapes fundamentally alter microbial interactions.

*Urbanization linked to structural, not functional aspects of stream ecosystems.*

Throughout my dissertation research, structural metrics describing both stream physicochemistry and microbial community structure were consistently related over space and time to watershed urbanization, but functional measurements were not. Organic carbon quality, bacterial community structure, nutrient fluxes and stoichiometry, and temperature were all key factors significantly linked to watershed urbanization. By contrast, functional measures of microbial enzyme activity were weakly, or not at all, correlated to catchment urbanization. Of the seven enzymes studied only one, phosphatase, was significantly correlated to watershed impervious cover. Further, bioavailability work revealed that microbial degradation of organic matter was greater in urbanized systems, but only during spring and summer months.

Predicting microbial functions based on structural attributes of an ecosystem is now recognized as a substantial challenge facing ecosystem ecologists. Such information is vital for researchers seeking to scale-up measures of microbial activity, such as EEA, in order to assess the cumulative impact of streams on ecosystem fluxes; yet, a review I conducted of published stream EEA studies (Table 2.7) shows that these efforts frequently report conflicting results on the variables controlling enzyme activity. This

evidence suggests that underlying factors controlling microbial EEA in streams have not yet been identified.

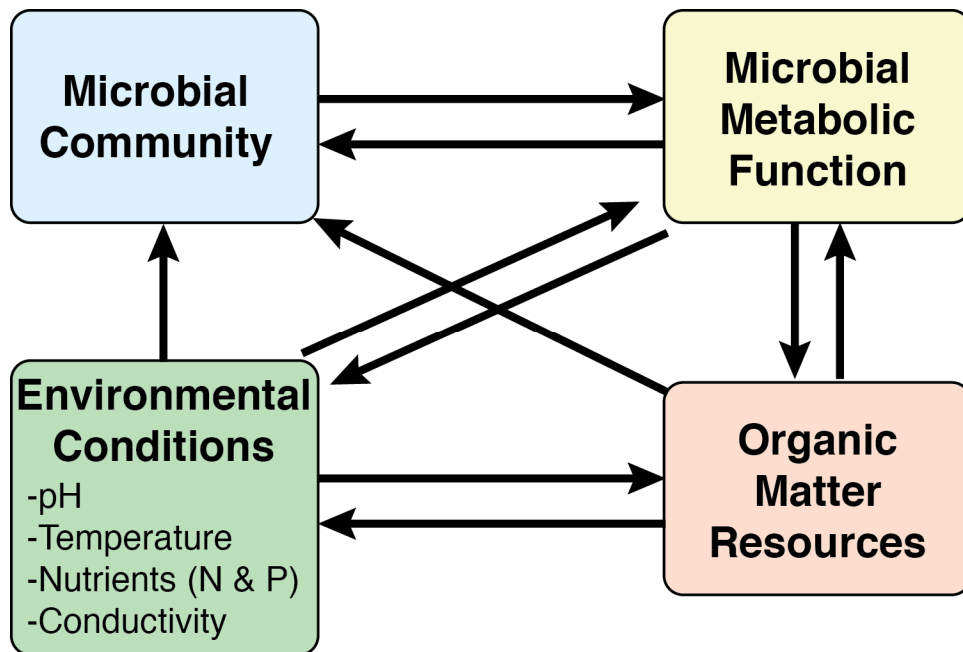
The underlying factors shaping microbial function are also hidden, in part because the manner in which complex microbial communities interact in the environment remains a black box. Most measurements of microbial metabolic activity in the environment are made on aggregate communities. This ignores the community interactions and competing processes that occur within biofilms (Battin et al., 2007). This is certainly true of EEA measures where production of a particular enzyme by microbes is based on both microbial demand and the availability of the enzyme's substrate in the environment (Sinsabaugh and Moorhead, 1994; Hill et al., 2010b; Sinsabaugh and Follstad Shah, 2012).

#### *Modeling stream microbial activity*

Models that take a mechanistic, system-based approach hold promise to more realistically capture the many interacting mechanisms driving microbial function. Such work begins with effective conceptual models that can serve as the basis for future mechanistic studies. Based on the research I present here and the literature reviewed for this dissertation, I have developed a conceptual diagram that can be the basis for future hypotheses and models exploring the relationship between microbes and organic matter resources in streams (Figure C.1).

With this model, I highlight the highly interconnected nature of the different elements of the microbial carbon cycle in streams. Clearly microbial communities exert influence on stream organic matter resources, however the reverse is also true. Microbial community structure is strongly influenced by the quality of organic matter available (Crump et al., 2003, 2003; Judd et al., 2006, 2007; Besemer et al., 2013). This effect can then feedback on organic matter resources as shifts in microbial community structure may alter the organic matter processing rate of that community (Reed and Martiny, 2012; Zeglin et al., 2013). The precise mechanistic outcome of such feedbacks is unclear at this time. As a result, this conceptual model can be viewed as a starting point. As further details emerge about specific relationships between factors, so will the detail of the conceptual diagram. Research that incorporates and directly addresses these diverse microbial processes has the power to open the black box of microbial communities.

Figure



**Figure C.1:** A conceptual diagram of the relationship between a stream microbial community, metabolic function of that microbial community, the organic matter resources used by heterotrophic taxa, and environmental conditions such as pH, temperature, and nutrient levels.

## Appendix I

A table of the humic index (HIX), fluorescence index (FI), and fluorescence EEM

PARAFAC scores of each sample included in Chapter 1.

Site	Date	HIX	FI	FMax_C1	FMax_C2	FMax_C3	FMax_C4	FMax_C5
F1	4/21/11	11.34336	1.40127	0.04538	0.02549	0.02183	0.01054	0.01029
F1	5/11/11	17.59559	1.40616	0.04927	0.02781	0.02285	0.00564	0.00688
F1	6/28/11	27.00663	1.40719	0.10837	0.06179	0.05009	0.01167	0.00928
F1	9/4/11	24.11064	1.43801	0.09811	0.05598	0.04871	0.00673	0.00777
F1	9/29/11	15.29866	1.39698	0.09715	0.05726	0.05060	0.01070	0.02196
F1	11/6/11	12.55397	1.48800	0.06211	0.03351	0.03222	0.01142	0.00638
F1	12/15/11	23.20925	1.50580	0.03893	0.01954	0.01934	0.00390	0.00396
F1	2/1/12	3.95339	1.46842	0.03220	0.01734	0.01839	0.04141	0.00600
F1	2/28/12	3.94975	1.48999	0.03271	0.01793	0.01890	0.04569	0.00142
F1	4/4/12	35.62158	1.44888	0.05006	0.02871	0.02290	0.00217	0.00519
F1	4/25/12	28.45682	1.43620	0.04949	0.02850	0.02407	0.00222	0.00489
F1	5/29/12	13.07229	1.39627	0.08393	0.05187	0.04145	0.02960	0.00610
F1	7/5/12	6.89347	1.41669	0.12848	0.07268	0.07912	0.08736	0.02317
F1	11/12/12	34.37053	1.45921	0.07436	0.03919	0.04297	0.01591	0.00859
F1	12/14/12	18.96475	1.44475	0.07095	0.04001	0.03801	0.00864	0.00610
F1	2/4/13	18.88439	1.46549	0.03879	0.02125	0.02089	0.00563	0.00271
F2	4/21/11	6.04813	1.33785	0.03522	0.02112	0.01706	0.01762	0.01035
F2	5/11/11	11.34032	1.33992	0.03184	0.01907	0.01353	0.00814	0.00505
F2	6/28/11	28.09214	1.32343	0.04153	0.02499	0.01715	0.00753	0.00350
F2	7/26/11	16.30845	1.33517	0.04461	0.02692	0.02132	0.00947	0.00653
F2	9/29/11	29.65404	1.35807	0.05901	0.03505	0.02895	0.00439	0.00643
F2	11/6/11	25.43064	1.38198	0.02643	0.01462	0.01289	0.01905	0.00183
F2	12/15/11	23.29558	1.36605	0.03236	0.01739	0.01434	0.00261	0.00458
F2	2/1/12	12.42686	1.34629	0.02611	0.01517	0.01222	0.00806	0.00291
F2	2/28/12	9.44240	1.34445	0.02484	0.01476	0.01204	0.01155	0.00303
F2	4/4/12	32.39563	1.33333	0.03188	0.01963	0.01361	0.00303	0.00243
F2	4/25/12	32.89079	1.31084	0.03634	0.02384	0.01583	0.00131	0.00503
F2	5/29/12	4.82952	1.31243	0.04449	0.02961	0.02716	0.05777	0.00145
F2	8/12/12	21.79270	1.34004	0.05012	0.03086	0.02515	0.00671	0.00692
F2	9/12/12	10.23992	1.35468	0.03718	0.02215	0.01829	0.01433	0.00407
F2	9/28/12	10.28184	1.35318	0.03391	0.02028	0.01615	0.01139	0.00457
F2	11/12/12	4.09619	1.37343	0.03692	0.02190	0.01955	0.04761	0.00490
F2	12/14/12	11.34767	1.38205	0.02710	0.01601	0.01311	0.00900	0.00182

Site	Date	HIX	FI	FMax_C1	FMax_C2	FMax_C3	FMax_C4	FMax_C5
F2	2/4/13	7.27660	1.32813	0.02187	0.01384	0.01105	0.01483	0.00137
F3	3/3/11	3.96880	1.43417	0.01671	0.00991	0.00959	0.01459	0.00748
F3	4/21/11	34.10032	1.39583	0.02722	0.01536	0.01241	0.00046	0.00322
F3	5/11/11	13.34867	1.40987	0.02301	0.01306	0.01198	0.00787	0.00313
F3	6/28/11	18.20414	1.42354	0.02321	0.01369	0.01056	0.00385	0.00207
F3	7/26/11	39.04082	1.39492	0.05341	0.02951	0.02436	0.00134	0.00796
F3	9/4/11	93.19787	1.38303	0.03531	0.01978	0.01649	0.00000	0.00293
F3	9/29/11	54.51495	1.39168	0.04639	0.02579	0.02101	0.00000	0.00255
F3	11/6/11	16.73045	1.43358	0.02764	0.01442	0.01451	0.00790	0.00505
F3	12/15/11	4.33320	1.43947	0.02925	0.01680	0.02181	0.04152	0.00061
F3	2/1/12	10.72640	1.42332	0.02394	0.01317	0.01234	0.00878	0.00276
F3	2/28/12	2.13464	1.42730	0.02447	0.01453	0.01821	0.07301	0.00178
F3	4/25/12	9.12686	1.40839	0.03257	0.01910	0.01801	0.01763	0.00312
F3	5/29/12	28.39896	1.38322	0.03884	0.02389	0.01923	0.00366	0.00379
F3	7/5/12	3.92321	1.39990	0.04245	0.02560	0.03178	0.06408	0.00365
F3	8/12/12	5.48547	1.40155	0.04455	0.02812	0.03322	0.04665	0.00240
F3	9/12/12	9.38427	1.41981	0.04383	0.02521	0.02199	0.02012	0.00429
F3	9/28/12	4.73392	1.71029	0.03689	0.01834	0.02257	0.00587	0.00767
F3	11/12/12	12.20949	1.40885	0.03288	0.01908	0.01448	0.00925	0.00456
F3	12/14/12	8.57268	1.43065	0.02681	0.01464	0.01400	0.01109	0.00344
F3	2/4/13	11.67627	1.40309	0.02230	0.01278	0.01259	0.00735	0.00076
F4	3/2/11	23.17937	1.34063	0.03406	0.01960	0.01576	0.00418	0.00363
F4	5/12/11	10.26956	1.33887	0.04304	0.02676	0.01939	0.01776	0.00504
F4	6/28/11	23.91801	1.28990	0.06578	0.04409	0.02714	0.01323	0.00484
F4	9/5/11	78.91661	1.34497	0.07228	0.04441	0.03256	0.00397	0.00221
F4	9/30/11	42.65613	1.33884	0.07340	0.04340	0.03272	0.00530	0.00519
F4	11/6/11	21.06358	1.35290	0.04465	0.02597	0.02007	0.00650	0.00177
F4	1/31/12	17.95311	1.32586	0.03264	0.01616	0.01489	0.00537	0.00301
F4	2/27/12	18.94082	1.30475	0.03357	0.01749	0.01524	0.00466	0.00298
F4	4/3/12	6.80613	1.32210	0.04070	0.02521	0.01968	0.02980	0.00265
F4	4/25/12	14.14472	1.30440	0.04872	0.03023	0.02475	0.01692	0.00431
F4	5/29/12	26.05701	1.29378	0.06887	0.04842	0.03141	0.01384	0.00430
F4	7/4/12	16.87346	1.30787	0.09179	0.05936	0.04588	0.02349	0.00643
F4	9/12/12	16.46634	1.31737	0.08303	0.05293	0.03703	0.01537	0.01026
F4	11/12/12	99.53820	1.35218	0.06015	0.03514	0.02663	0.00000	0.00208
F4	12/14/12	26.48250	1.35723	0.04946	0.03066	0.02311	0.00584	0.00130
F4	2/5/13	21.36595	1.36270	0.03865	0.02322	0.01956	0.00699	0.00095
F5	3/2/11	13.43941	1.36171	0.02630	0.01594	0.01310	0.00454	0.00280
F5	5/12/11	12.25217	1.35894	0.02974	0.01821	0.01226	0.00883	0.00436
F5	6/28/11	9.85653	1.31401	0.06365	0.04115	0.02281	0.03610	0.00468
F5	9/30/11	22.50552	1.38056	0.04817	0.02842	0.02062	0.00509	0.00444
F5	11/6/11	7.26832	1.36901	0.03539	0.02131	0.01620	0.02247	0.00203



Site	Date	HIX	FI	FMax_C1	FMax_C2	FMax_C3	FMax_C4	FMax_C5
F5	12/14/11	20.86577	1.33264	0.03274	0.01467	0.01454	0.00448	0.00304
F5	1/31/12	13.18557	1.39307	0.02916	0.01350	0.01189	0.00785	0.00150
F5	2/27/12	7.46472	1.30614	0.03080	0.01695	0.01524	0.02077	0.00210
F5	4/3/12	6.73867	1.36742	0.02843	0.01741	0.01383	0.01962	0.00236
F5	4/25/12	21.35727	1.36168	0.04431	0.02686	0.01934	0.00801	0.00405
F5	5/29/12	27.90602	1.30314	0.06765	0.04578	0.02922	0.01335	0.00392
F5	8/12/12	16.16618	1.27510	0.10263	0.06791	0.04878	0.03935	0.01114
F5	11/12/12	52.87747	1.38665	0.04430	0.02631	0.02010	0.00226	0.00165
F5	12/14/12	16.08699	1.35586	0.04190	0.02529	0.01939	0.00908	0.00174
F5	2/5/13	15.76251	1.36579	0.03510	0.02138	0.01738	0.00873	0.00163
U1	3/2/11	9.02862	1.55977	0.02646	0.00926	0.02122	0.00593	0.00900
U1	4/21/11	6.37533	1.54128	0.04292	0.01621	0.03210	0.02061	0.01708
U1	5/11/11	7.71199	1.56292	0.03225	0.01209	0.02586	0.00609	0.01547
U1	6/28/11	8.85551	1.59159	0.03033	0.01027	0.02474	0.00706	0.01302
U1	7/26/11	11.51505	1.57451	0.03524	0.01333	0.02976	0.00503	0.01409
U1	9/4/11	12.79809	1.50679	0.03193	0.01020	0.02577	0.02699	0.02403
U1	9/30/11	12.20551	1.54394	0.05139	0.01849	0.03685	0.00881	0.02110
U1	11/6/11	5.39757	1.60194	0.02694	0.00888	0.02426	0.02052	0.01115
U1	12/14/11	5.75240	1.62455	0.02358	0.00799	0.02013	0.01991	0.00463
U1	1/31/12	6.64252	1.58567	0.02685	0.00926	0.02137	0.01551	0.00861
U1	2/27/12	6.62761	1.55584	0.02777	0.00981	0.02259	0.01694	0.00832
U1	4/3/12	12.64586	1.54573	0.04604	0.01777	0.03344	0.00786	0.01425
U1	4/25/12	5.05122	1.57469	0.03848	0.01523	0.03437	0.04172	0.00849
U1	5/29/12	10.97324	1.48355	0.06712	0.02836	0.05037	0.01488	0.02297
U1	7/4/12	3.57819	1.58059	0.04970	0.01835	0.05165	0.07120	0.01786
U1	8/12/12	10.45390	1.59816	0.03728	0.01201	0.03708	0.00691	0.01512
U1	9/12/12	7.65411	1.61263	0.02973	0.00943	0.02693	0.01120	0.01032
U1	9/28/12	9.91593	1.60694	0.03340	0.01057	0.03033	0.00732	0.01127
U1	11/12/12	13.14184	1.63802	0.03275	0.01023	0.02850	0.00123	0.01108
U1	12/14/12	7.31480	1.62588	0.03007	0.01001	0.02526	0.01324	0.00943
U1	2/4/13	21.55246	1.58528	0.02485	0.00808	0.02112	0.00132	0.00464
U2	3/2/11	12.29157	1.57345	0.03724	0.01409	0.02536	0.00463	0.00780
U2	5/11/11	3.60272	1.59408	0.03002	0.01098	0.02590	0.02380	0.02055
U2	6/28/11	5.16218	1.58866	0.04730	0.01747	0.03946	0.02074	0.02810
U2	7/26/11	5.99184	1.56933	0.05190	0.01912	0.04335	0.01438	0.03198
U2	9/4/11	7.65889	1.59231	0.03895	0.01481	0.02978	0.00931	0.01412
U2	9/30/11	11.45131	1.58457	0.04390	0.01661	0.03070	0.00876	0.00900
U2	11/6/11	4.89601	1.62898	0.03366	0.01230	0.02598	0.02176	0.00823
U2	12/14/11	10.70580	1.62701	0.03482	0.01407	0.02453	0.01346	0.00371
U2	1/31/12	6.29074	1.61586	0.03217	0.01214	0.02401	0.02109	0.00541
U2	2/27/12	7.76873	1.61296	0.03360	0.01268	0.02501	0.01515	0.00685
U2	4/3/12	12.50038	1.54373	0.04303	0.01685	0.03053	0.00140	0.01450

Site	Date	HIX	FI	FMax_C1	FMax_C2	FMax_C3	FMax_C4	FMax_C5
U2	4/25/12	12.39943	1.56989	0.04791	0.01864	0.03394	0.00610	0.01309
U2	5/29/12	7.91348	1.54418	0.05747	0.02292	0.04472	0.01571	0.02180
U2	7/4/12	5.49359	1.56948	0.06361	0.02413	0.05374	0.02433	0.03855
U2	9/12/12	5.76188	1.57525	0.06508	0.02471	0.05380	0.01930	0.03810
U2	11/12/12	11.59178	1.58376	0.05418	0.02094	0.03702	0.00928	0.01281
U2	12/14/12	10.13742	1.55613	0.05002	0.02033	0.03348	0.01435	0.00708
U2	2/4/13	11.45693	1.55125	0.04290	0.01635	0.03212	0.00902	0.01116
U3	3/2/11	4.64285	1.59013	0.01316	0.00464	0.01047	0.00796	0.00646
U3	4/21/11	23.39615	1.50881	0.01712	0.00635	0.01196	0.00000	0.01478
U3	5/11/11	2.72547	1.51177	0.01664	0.00687	0.01657	0.02150	0.01661
U3	6/28/11	5.77862	1.52420	0.01766	0.00697	0.01293	0.01368	0.00753
U3	7/26/11	14.81870	1.51874	0.02542	0.00981	0.01968	0.00155	0.00938
U3	9/4/11	18.09619	1.50065	0.02346	0.00897	0.01811	0.00000	0.00723
U3	9/30/11	7.80096	1.51559	0.02342	0.00879	0.01930	0.00000	0.01203
U3	1/31/12	1.08346	1.64874	0.01120	0.00499	0.01963	0.07623	0.00446
U3	2/27/12	5.34863	1.63883	0.01375	0.00437	0.01094	0.00767	0.00598
U3	4/3/12	9.61201	1.57519	0.01678	0.00576	0.01213	0.00009	0.00928
U3	4/25/12	4.12259	1.53507	0.02096	0.00799	0.01868	0.02560	0.00910
U3	5/29/12	3.17834	1.54747	0.01964	0.00828	0.02143	0.03280	0.00769
U3	7/4/12	3.38907	1.53244	0.02037	0.00787	0.02055	0.02760	0.00907
U3	8/12/12	2.74712	1.52909	0.02339	0.01014	0.02802	0.04900	0.00799
U3	9/12/12	8.17285	1.53089	0.02592	0.00973	0.02160	0.00854	0.00916
U3	9/28/12	8.53944	1.52992	0.02129	0.00823	0.01761	0.00743	0.00480
U3	11/12/12	7.61697	1.58036	0.02073	0.00668	0.01468	0.00412	0.00706
U3	12/14/12	6.74759	1.54653	0.01627	0.00567	0.01388	0.00678	0.00517
U3	2/4/13	4.42063	1.56511	0.01887	0.00667	0.01851	0.01102	0.01242

## Appendix II

Mean EEA values by site for the two seasonal groups identified, Spring/Summer and Fall/Winter. Standard error of the mean is presented in parenthesis.

Site	Esterase		LAPase		$\alpha$ Gase		$\beta$ Gase		NAGase		XYLase	
	Spring/Summer	Fall/Winter	Spring/Summer	Fall/Winter	Spring/Summer	Fall/Winter	Spring/Summer	Fall/Winter	Spring/Summer	Fall/Winter	Spring/Summer	Fall/Winter
F1	1603.4 (1531.6)	1788.4 (256.1)	179.6 (141.5)	154.2 (9.3)	3.05 (1.22)	2.86 (1.48)	7.37 (2.28)	6.19 (2.88)	5.64 (0.68)	5.34 (1.44)	2.43 (0.80)	2.45 (0.79)
F6	1551.8 (472.7)	986.1 (56.1)	110.8 (15.0)	108.6 (4.5)	5.97 (3.66)	2.46 (0.50)	8.38 (3.19)	9.52 (3.67)	11.5 (3.79)	4.50 (0.81)	2.34 (0.70)	2.38 (0.39)
F2	1262.5 (722.2)	202.1 (20.8)	109.7 (27.1)	94.5 (20.0)	3.31 (1.01)	4.58 (1.97)	10.2 (2.80)	11.6 (3.64)	11.3 (4.63)	5.38 (1.04)	2.27 (0.99)	4.10 (2.06)
F3	1242.1 (382.8)	833.1 (94.9)	77.1 (11.0)	95.5 (14.6)	2.25 (1.15)	1.81 (0.66)	9.46 (3.35)	7.96 (1.77)	11.4 (1.51)	5.39 (1.65)	1.62 (0.44)	2.74 (0.68)
F4	135.3 (82.0)	102.3 (19.3)	40.0 (11.8)	71.4 (28.0)	1.29 (0.57)	7.16 (3.14)	12.9 (3.98)	20.3 (6.50)	6.92 (2.07)	4.82 (0.60)	0.98 (0.10)	8.81 (5.00)
F5	1568.8 (1207.2)	73.7 (11.1)	169.4 (103.4)	57.0 (19.5)	10.8 (6.11)	1.94 (0.34)	19.7 (7.55)	10.1 (3.29)	13.0 (2.27)	4.93 (0.59)	23.4 (20.4)	2.22 (0.72)
F7	2030.1 (870.2)	522.9 (77.9)	180.3 (64.5)	95.0 (7.0)	5.52 (1.31)	2.94 (0.66)	13.4 (3.12)	18.6 (12.2)	25.6 (7.90)	5.76 (1.41)	3.82 (0.85)	3.54 (2.16)
U4	1252.6 (324.8)	569.0 (52.2)	107.4 (10.4)	166.8 (68.8)	2.28 (0.89)	5.15 (3.10)	14.9 (11.9)	13.8 (2.92)	5.82 (0.61)	3.35 (0.60)	1.06 (0.33)	5.39 (0.01)
U1	660.7 (339.3)	118.1 (12.5)	82.7 (41.2)	86.1 (45.3)	4.38 (1.42)	6.79 (4.41)	5.41 (2.57)	6.44 (2.99)	5.56 (1.91)	4.19 (1.26)	1.12 (0.74)	2.04 (1.00)
U2	1284.4 (250.9)	242.2 (20.2)	186.2 (84.9)	109.2 (28.6)	9.49 (5.97)	3.45 (1.24)	20.5 (17.8)	25.4 (15.9)	7.64 (4.14)	14.1 (5.68)	5.17 (4.67)	18.1 (11.3)
U3	1002.7 (331.5)	304.8 (58.0)	193.3 (53.9)	169.9 (49.7)	8.42 (3.13)	5.23 (1.25)	14.5 (5.51)	22.8 (11.3)	8.35 (1.61)	7.72 (1.37)	3.86 (2.27)	5.67 (2.00)

### Appendix III

A summary of  $E_a$  values obtained from this study as well as other studies from freshwater and terrestrial ecosystems.

Enzyme	Study	$E_a$ (eV)	Environment	Habitat
Alkaline Phosphatase	(Menichetti et al., 2014)	0.084	Oaxaca Coffee Plantation Soil Dry	Soil
Alkaline Phosphatase	(Menichetti et al., 2014)	0.169	Oaxaca Coffee Plantation Soil Wet	Soil
Alkaline Phosphatase	(Menichetti et al., 2014)	0.138	Oaxaca Forest Soil Dry	Soil
Alkaline Phosphatase	(Menichetti et al., 2014)	0.112	Oaxaca Forest Soil Wet	Soil
Alkaline Phosphatase	(Menichetti et al., 2014)	0.055	Puebla Soil Dry	Soil
Alkaline Phosphatase	(Menichetti et al., 2014)	0.269	Puebla Soil Wet	Soil
Alkaline Phosphatase	(Sinsabaugh and Shah, 2010)	0.480	Ottawa and Maumee Rivers	River Water Column
Alkaline Phosphatase	This Study	0.232	Parkers Creek Water Column	Stream Water Column
Alkaline Phosphatase	(Wilczek et al., 2005)	0.440	River Sediment	River Sediment
Alkaline Phosphatase	(Ylla et al., 2014)	0.860	Stream Biofilm Mesocosm	Stream Biofilm
alpha-glucosidase	(Sinsabaugh and Shah, 2010)	0.030	Ottawa and Maumee Rivers	River Water Column
alpha-glucosidase	(Stone et al., 2012)	0.381	Bear Brook Soil	Soil
alpha-glucosidase	(Menichetti et al., 2014)	0.318	Fernow Soil	Soil
alpha-glucosidase	(Wilczek et al., 2005)	0.520	River Sediment	River Sediment
beta-glucosidase	(Davidson et al., 2012)	0.610	Mineral Soil	Soil
beta-glucosidase	(Davidson et al., 2012)	0.670	Organic Soil	Soil
beta-glucosidase	(Kähkönen et al., 2001)	0.580	Scotts Pine Forest Soil, Finland	Soil
beta-glucosidase	(Sinsabaugh and Shah, 2010)	0.120	Ottawa and Maumee Rivers	River Water Column
beta-glucosidase	(Steinweg et al., 2013)	0.370	Arctic Active Soil	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.360	Arctica Permafrost Soil	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.370	Subarctic A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.540	Subarctic B Horizon	Soil

beta-glucosidase	(Steinweg et al., 2013)	0.420	Temperate 1 A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.510	Temperate 1 B Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.320	Temperate 2 A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.420	Temperate 2 B Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.530	Temperate 3 A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.610	Temperate 3 B Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.500	Tropical 1 A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.590	Tropical 1 B Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.410	Tropical 2 A Horizon	Soil
beta-glucosidase	(Steinweg et al., 2013)	0.440	Tropical 2 B Horizon	Soil
beta-glucosidase	(Stone et al., 2012)	0.406	Bear Brook Soil	Soil
beta-glucosidase	(Stone et al., 2012)	0.378	Fernow Soil	Soil
beta-glucosidase	This Study	0.921	Parkers Creek Water Column	Stream Water Column
beta-glucosidase	(Trasar-Cepeda et al., 2007)	0.270	Navia Soil	Soil
beta-glucosidase	(Trasar-Cepeda et al., 2007)	0.310	Pontareas Soil	Soil
beta-glucosidase	(Trasar-Cepeda et al., 2007)	0.300	Sobrado Soil	Soil
beta-glucosidase	(Wilczek et al., 2005)	0.390	River Sediment	River Sediment
beta-glucosidase	(Ylla et al., 2014)	1.350	Stream Biofilm Mesocosm	Stream Biofilm
beta-xylosidase	(Stone et al., 2012)	0.439	Bear Brook Soil	Soil
beta-xylosidase	(Stone et al., 2012)	0.419	Fernow Soil	Soil
beta-xylosidase	(Ylla et al., 2014)	1.610	Stream Biofilm Mesocosm	Stream Biofilm
Esterase (acetate)	(Kähkönen et al., 2001)	0.530	Scotts Pine Forest Soil, Finland	Soil
leucine-aminopeptidase	(Sinsabaugh and Shah, 2010)	0.610	Ottawa and Maumee Rivers	Stream Water Column
leucine-aminopeptidase	(Wilczek et al., 2005)	0.460	River Sediment	River Sediment
leucine-aminopeptidase	(Ylla et al., 2014)	1.930	Stream Biofilm Mesocosm	Stream Biofilm
n-acetyl-glucoasaminidase	(Stone et al., 2012)	0.402	Bear Brook Soil	Soil
n-acetyl-glucoasaminidase	(Stone et al., 2012)	0.446	Fernow Soil	Soil

## Appendix IV

Class-level percent composition of top ten taxa as measured by 16S rDNA of each sample included in Chapter 3 community analysis.

Site	F1	F1	F1	F1	F1	F1	F1	F2
Date	1/31/12	4/23/12	8/12/12	11/12/12	11/12/12	2/4/13	2/4/13	1/31/12
Matrix	Water	Water	Sediment	Sediment	Water	Sediment	Water	Water
<b>Epsilonproteobacteria</b>	1.704	2.144	0.128	0.512	1.656	0.076	1.592	2.12
<b>Actinobacteria</b>	0.648	0.608	0.588	1.012	0.244	0.572	0.884	1.092
<b>Cytophagia</b>	2.396	2.184	1.332	0.832	4.388	0.828	1.312	3.472
<b>OPB35_soil_group</b>	2.868	1.692	2.612	2.756	0.5	3.136	0.884	1.276
<b>Acidobacteria</b>	3.868	1.816	6.62	6.724	0.7	7.704	1.084	1.252
<b>Sphingobacteriia</b>	6.172	4.932	3.04	4.004	2.156	2.252	4.52	7.168
<b>Alphaproteobacteria</b>	10.204	8.056	8.8	10.868	5.368	9.636	7.7	9.64
<b>Deltaproteobacteria</b>	9.952	9.612	18.064	14.78	6.896	17.628	7.464	6.784
<b>Gammaproteobacteria</b>	10.944	12.084	8.556	7.86	15.436	7.84	15.364	16.564
<b>Betaproteobacteria</b>	17.652	12.772	14.168	15.196	23.208	14.324	11.652	14.956
<b>Unclassified</b>	13.46	23.976	7.848	7.304	23.788	7.728	27.952	19.008
<b>Other</b>	20.132	20.124	28.244	28.152	15.66	28.276	19.592	16.668

Site	F2	F2	F2	F2	F2	F2	F7	F7
Date	4/23/12	8/12/12	8/12/12	11/12/12	2/4/13	2/4/13	1/31/12	4/23/12
Matrix	Water	Sediment	Water	Water	Sediment	Water	Water	Water
<b>Epsilonproteobacteria</b>	2.38	0.172	1.636	2.296	0.304	3.444	2.132	1.22
<b>Actinobacteria</b>	0.68	2.764	0.556	0.468	2.096	0.788	0.632	0.512
<b>Cytophagia</b>	5.888	0.588	1.296	12.52	1.216	1.608	2.48	4.04
<b>OPB35_soil_group</b>	0.972	2.864	2.48	0.608	2.88	2.588	1.504	3.04
<b>Acidobacteria</b>	0.804	7.112	3.116	0.872	5.908	1.96	2.224	3.156
<b>Sphingobacteriia</b>	3.7	3.916	4.584	4.88	5.936	6.564	6.964	8.556
<b>Alphaproteobacteria</b>	7.068	15.92	12.656	6.9	14.712	11.048	11.172	11.356
<b>Deltaproteobacteria</b>	6.348	14.368	12.124	7.22	14.496	7.432	8.756	10.688
<b>Gammaproteobacteria</b>	13.936	8.864	19.824	8.292	8.876	14.916	13.36	11.352
<b>Betaproteobacteria</b>	15.072	11.692	16.996	36.2	13.968	15.372	18.716	20.636
<b>Unclassified</b>	23.984	5.82	8.132	10.024	6.332	17.544	14.06	8.168
<b>Other</b>	19.168	25.92	16.6	9.72	23.276	16.736	18	17.276

Site	F7	F7	F7	F7	F7	F3	F3	F3
Date	8/12/12	11/12/12	11/12/12	2/4/13	2/4/13	1/31/12	4/23/12	8/12/12
Matrix	Sediment	Sediment	Water	Sediment	Water	Water	Water	Sediment
<b>Epsilonproteobacteria</b>	0.204	0.092	1.18	0.26	1.056	1.088	1.928	0.132
<b>Actinobacteria</b>	0.472	0.524	0.528	1.784	0.608	0.504	0.616	0.456
<b>Cytophagia</b>	1.564	1.832	4.928	1.56	2.932	3.236	3.248	1.096
<b>OPB35_soil_group</b>	2.444	2.048	1.008	1.704	2.116	3.18	2.3	2.336
<b>Acidobacteria</b>	5.808	4.584	1.028	4.98	2.136	3.744	2.9	6.076
<b>Sphingobacteriia</b>	4.984	5.152	5.428	5.84	7.356	7.776	6.46	4.912
<b>Alphaproteobacteria</b>	8.352	10.172	11.568	15.076	10.928	11.54	10.752	7.868
<b>Deltaproteobacteria</b>	14.128	14.716	8.408	12.604	8.516	10.432	8.596	12.096
<b>Gammaproteobacteria</b>	12.992	12.632	13.864	14.824	17.948	13.636	11.264	20.496
<b>Betaproteobacteria</b>	15.312	20.204	31.152	15.696	16.876	21.308	19.448	17.484
<b>Unclassified</b>	8.44	6.392	7.484	5.8	13.228	7.712	13.44	6.952
<b>Other</b>	25.3	21.652	13.424	19.872	16.3	15.844	19.048	20.096



Site	F3	F3	F3	F3	F4	F4	F4	F4
Date	8/12/12	11/12/12	11/12/12	2/4/13	1/31/12	4/23/12	8/12/12	11/12/12
Matrix	Water	Sediment	Water	Water	Water	Water	Sediment	Sediment
<b>Epsilonproteobacteria</b>	1.52	0.26	1.596	2.184	2.208	3.508	0.04	0.208
<b>Actinobacteria</b>	0.5	0.38	0.24	0.612	0.428	0.448	0.732	0.72
<b>Cytophagia</b>	1.448	1.372	3.696	2.42	1.12	2.332	1.528	1.472
<b>OPB35_soil_group</b>	2.844	1.836	1	2.488	5.776	3.304	4.672	4.568
<b>Acidobacteria</b>	4.572	4.152	1.028	1.844	11.688	4.428	11.78	9.172
<b>Sphingobacteriia</b>	4.772	5.288	5.276	7.004	4.708	5.072	4.172	5.236
<b>Alphaproteobacteria</b>	10.664	9.716	8.108	10.172	13.86	9.716	11.576	12.572
<b>Deltaproteobacteria</b>	10.972	12.176	9.516	9.068	10.056	7.964	14.86	11.52
<b>Gammaproteobacteria</b>	14.916	20.844	17.012	18.448	7.516	12.932	5.88	4.832
<b>Betaproteobacteria</b>	20.708	19.2	29.34	14.908	11.812	13.76	12.04	18.012
<b>Unclassified</b>	9.344	6.344	9.352	13.492	8.492	16.532	6.396	5.732
<b>Other</b>	17.74	18.432	13.836	17.36	22.336	20.004	26.324	25.956

Site	F4	F4	F4	F5	F5	F5	F5	F5
Date	11/12/12	2/4/13	2/4/13	1/31/12	8/12/12	8/12/12	11/12/12	11/12/12
Matrix	Water	Sediment	Water	Water	Sediment	Water	Sediment	Water
<b>Epsilonproteobacteria</b>	2.304	0.656	2.948	3.088	0.068	1.524	0.116	2.036
<b>Actinobacteria</b>	0.18	1.112	0.372	0.564	2.276	0.428	2.028	0.292
<b>Cytophagia</b>	2.656	1.772	2.372	1.508	0.796	1.276	1.832	4.38
<b>OPB35_soil_group</b>	1.596	5.044	4.88	3.64	3.532	3.444	4.032	2.328
<b>Acidobacteria</b>	2.3	9.02	6.676	3.62	7.42	2.604	7.328	3.56
<b>Sphingobacteriia</b>	5.864	4.632	7.944	6.312	4.368	4.164	5.556	9.708
<b>Alphaproteobacteria</b>	11.248	14.18	15.128	14.564	17.476	10.168	19.628	14.312
<b>Deltaproteobacteria</b>	5.248	14.872	8.412	7.348	13.368	8.02	11.192	4.948
<b>Gammaproteobacteria</b>	7.072	5.312	9.116	14.968	9.82	22.752	7.916	10.056
<b>Betaproteobacteria</b>	45.064	14.192	18.724	14.508	12.292	28.736	16.252	36.132
<b>Unclassified</b>	6.048	5.34	7.104	14.192	5.556	5.54	5.124	3.996
<b>Other</b>	10.42	23.868	16.324	15.688	23.028	11.344	18.996	8.252

Site	F5	F5	F7	F7	F7	F7	F7	F7
Date	2/4/13	2/4/13	1/31/12	4/23/12	8/12/12	11/12/12	11/12/12	2/4/13
Matrix	Sediment	Water	Water	Water	Sediment	Sediment	Water	Sediment
<b>Epsilonproteobacteria</b>	0.22	3.332	2.812	2.572	0.232	0.756	2.476	1.016
<b>Actinobacteria</b>	1.204	0.492	0.476	0.532	0.972	0.576	0.172	1.076
<b>Cytophagia</b>	1.684	1.588	1.832	3.196	1.736	1.712	3.504	1.532
<b>OPB35_soil_group</b>	3.976	4.684	2.416	2.98	2.856	2.244	0.692	2.504
<b>Acidobacteria</b>	7.544	4.896	3.684	4.296	5.592	5.156	0.632	5.836
<b>Sphingobacteriia</b>	4.492	6.884	6.356	7.48	6.888	6.992	4.536	6.5
<b>Alphaproteobacteria</b>	14.688	14.776	12.216	9.444	7.628	9.584	6.74	12.568
<b>Deltaproteobacteria</b>	14.2	8.156	8.748	9.136	14.396	12.82	5.92	14.276
<b>Gammaproteobacteria</b>	7.936	12.772	10.92	8.5	13.676	10.896	6.668	9.936
<b>Betaproteobacteria</b>	12.44	14.428	20.16	17.528	16.904	23.648	45.356	16.72
<b>Unclassified</b>	6.94	11.304	12.608	13.704	6.756	5.372	7.644	5.884
<b>Other</b>	24.676	16.688	17.772	20.632	22.364	20.244	15.66	22.152

Site	F7	U4	U4	U4	U4	U4	U4	U4
Date	2/4/13	1/31/12	4/23/12	8/12/12	8/12/12	11/12/12	11/12/12	2/4/13
Matrix	Water	Water	Water	Sediment	Water	Sediment	Water	Sediment
<b>Epsilonproteobacteria</b>	3.444	1.564	1.816	0.028	2.184	0.464	1.404	0.18
<b>Actinobacteria</b>	0.368	1.392	4.64	1.104	2.356	0.4	0.636	0.428
<b>Cytophagia</b>	1.74	2.388	3.352	1.316	1.556	2.732	2.868	1.372
<b>OPB35_soil_group</b>	2.888	0.584	0.856	1.696	0.448	1.54	0.328	1.636
<b>Acidobacteria</b>	2.932	0.624	0.844	4.448	0.664	2.848	0.36	4.048
<b>Sphingobacteriia</b>	8.224	4.828	5.86	4.472	3.184	5.152	3.044	4.476
<b>Alphaproteobacteria</b>	9.444	5.412	4.884	11.316	9.108	9.624	4.476	6.284
<b>Deltaproteobacteria</b>	9.884	6.204	4.896	13.896	7.208	11.1	8.768	14.144
<b>Gammaproteobacteria</b>	12.24	12.164	9.868	12.836	16.268	18.628	14.164	22.936
<b>Betaproteobacteria</b>	16.552	26.312	28.92	16.072	25.344	24.932	39.8	17.568
<b>Unclassified</b>	11.664	22.644	16.152	7.784	14.72	5.404	8.608	6.72
<b>Other</b>	20.62	15.884	17.912	25.032	16.96	17.176	15.544	20.208

Site	U4	U1	U1	U1	U1	U1	U1	U1
Date	2/4/13	1/31/12	4/23/12	8/12/12	8/12/12	11/12/12	11/12/12	2/4/13
Matrix	Water	Water	Water	Sediment	Water	Sediment	Water	Sediment
<b>Epsilonproteobacteria</b>	0.556	3.284	2.324	0.012	1.676	0.352	1.732	0.16
<b>Actinobacteria</b>	19.832	0.52	0.596	0.952	0.36	0.824	2.672	0.86
<b>Cytophagia</b>	2.832	0.596	2.336	1.592	0.564	0.916	2.156	0.712
<b>OPB35_soil_group</b>	0.54	1.46	1.288	1.656	0.648	1.904	0.972	2.548
<b>Acidobacteria</b>	0.132	2.288	1.068	5.176	1.236	4.676	0.42	6.188
<b>Sphingobacteriia</b>	10.988	4.404	4	2.256	2.664	2.672	7.14	2.704
<b>Alphaproteobacteria</b>	2.988	5.244	4.196	11.76	6.844	9.616	4.604	11.22
<b>Deltaproteobacteria</b>	1.688	6.804	6.876	11.624	6.408	16.444	9.504	14.8
<b>Gammaproteobacteria</b>	3.952	13.424	15.472	23.244	22.936	12.38	12.584	12.496
<b>Betaproteobacteria</b>	39.272	19.656	18.076	17.468	24.032	15.532	31.056	13.104
<b>Unclassified</b>	12.048	23.896	23.296	5.288	17.448	6.752	11.576	6.976
<b>Other</b>	5.172	18.424	20.472	18.972	15.184	27.932	15.584	28.232

Site	U1	U2	U2	U2	U2	U2	U2	U2
Date	2/4/13	1/31/12	4/23/12	8/12/12	8/12/12	11/12/12	2/4/13	2/4/13
Matrix	Water	Water	Water	Sediment	Water	Water	Sediment	Water
<b>Epsilonproteobacteria</b>	9.448	3.328	1.736	0.124	1.488	3.1	0.332	4.212
<b>Actinobacteria</b>	0.456	0.436	8.216	1.82	0.272	0.292	1.428	0.404
<b>Cytophagia</b>	0.4	0.292	2.044	1.036	0.804	2.356	0.572	0.532
<b>OPB35_soil_group</b>	0.708	0.756	0.92	3.356	0.656	0.308	3.84	0.828
<b>Acidobacteria</b>	0.676	1.34	1.364	5.912	1.124	0.5	4.572	0.776
<b>Sphingobacteriia</b>	3.144	3.58	6.98	3.24	3.256	2.744	3.008	2.636
<b>Alphaproteobacteria</b>	4.472	6.728	5.196	10.548	6.092	3.404	7.672	4.772
<b>Deltaproteobacteria</b>	5.608	8.504	5.28	14.936	8.536	11.088	12.708	7.74
<b>Gammaproteobacteria</b>	17.08	11.056	9.1	11.224	13.8	15.04	22.012	16.916
<b>Betaproteobacteria</b>	18.868	16.384	24.788	12.492	29.26	27.908	19.168	18.412
<b>Unclassified</b>	23.956	28.18	19.136	7.884	18.368	19.268	6.248	28.224
<b>Other</b>	15.184	19.416	15.24	27.428	16.344	13.992	18.44	14.548

Site	U3	U3	U3	U3	U3	U3
Date	1/31/12	4/23/12	8/12/12	11/12/12	2/4/13	2/4/13
Matrix	Water	Water	Water	Sediment	Sediment	Water
<b>Epsilonproteobacteria</b>	1.612	0.884	1.3	0.684	1.224	3.232
<b>Actinobacteria</b>	3.544	21.268	6.74	0.628	1.008	4.3
<b>Cytophagia</b>	5.28	3.752	2.604	0.864	1.468	1.668
<b>OPB35_soil_group</b>	0.844	0.776	0.848	3.008	3.496	0.624
<b>Acidobacteria</b>	0.768	1.476	1.08	9.2	6.776	0.636
<b>Sphingobacteriia</b>	7.868	7.672	2.7	3.396	7.212	7.352
<b>Alphaproteobacteria</b>	8.164	7.296	6.812	7.316	8.68	4.088
<b>Deltaproteobacteria</b>	4.9	2.592	6.544	15.1	13.308	5.948
<b>Gammaproteobacteria</b>	11.364	2.52	13.892	8.148	10.836	15.16
<b>Betaproteobacteria</b>	30.672	33.392	22.492	13.88	18.292	22.56
<b>Unclassified</b>	11.588	6.828	14.076	9.644	7.256	18.916
<b>Other</b>	13.396	11.544	20.912	28.132	20.444	15.516

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