SPATIO-TEMPORAL VARIATION AND DISSOLVED ORGANIC CARBON PROCESSING OF STREAMBED MICROBIAL COMMUNITY: STABLE CARBON ISOTOPE APPROACH

by

PHILIPS OLUGBEMIGA AKINWOLE

ROBERT H. FINDLAY, COMMITTEE CHAIR
AMELIA K. WARD
JULIE B. OLSON
BEHZAD MORTAZAVI
FRED T. ANDRUS

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biological Sciences in the Graduate School of The University of Alabama

TUSCALOOSA, ALABAMA

2013
ABSTRACT

Sedimentary microbial communities play a critical ecological role in lotic ecosystems and are responsible for numerous biogeochemical transformations, including dissolved organic matter (DOM) uptake, degradation, and mineralization. The goals of this study were to elucidate the benthic microbes responsible for utilization of humic DOM in streams and to assess overall variability in microbial biomass and community structure over time and across multiple spatial scales in stream networks, as DOM quality and quantity will likely change with stream order. In Chapter 2, multiple spatial patterns of microbial biomass and community structure were examined in stream sediments from two watersheds; the Neversink River watershed (NY; 1st, 3rd and 5th order streams sampled) and the White Clay Creek watershed (PA; 1st through 3rd order streams sampled). Microbial biomass and community structure were estimated by phospholipid phosphate and phospholipid fatty acids (PLFA) analyses. Multivariate analysis showed that sedimentary C:N ratios, percent carbon, sediment surface area and percent water content explained 68% of the variations in total microbial biomass. Overall, the magnitude of within stream variation in microbial biomass was small compared to the variability noted among streams and between watersheds. Principal component analysis (PCA) of PLFA profiles showed that microbial community structure displayed a distinct watershed-level biogeography, as well as variation along a stream order gradient. Chapter 3 demonstrated that benthic microbial biomass was seasonally dynamic and significantly correlated to a combination of high and low flood pulse counts, variability in daily flow and DOC concentration in the White Clay Creek. Additionally, the seasonal pattern of variation observed in microbial community structure was as
a result of shift between the ratios of prokaryotic to eukaryotic component of the community. This shift was significantly correlated with seasonal changes in median daily flow, high and low flood pulse counts, DOC concentrations and water temperature. Compound-specific $^{13}$C analysis of PLFA showed that both bacterial and microeukaryotic stable carbon isotope ratios were heaviest in the spring and lightest in autumn or winter. Bacterial lipids were isotopically depleted on average by 2 - 5‰ relative to $\delta^{13}$C of total organic carbon suggesting bacterial consumption of allochthonous organic matter, and enriched relative to $\delta^{13}$C algae-derived carbon source. In Chapter 4, heterotrophic microbes that metabolize humic DOM in a third-order stream were identified through trace-additions of $^{13}$C-labeled tree tissue leachate ($^{13}$C-DOC) into stream sediment mesocosms. Microbial community structure was assessed using PLFA biomarkers, and metabolically active members were identified through $^{13}$C-PLFA analysis (PLFA-SIP). Comparison by PCA of the microbial communities in stream sediments and stream sediments incubated in both the presence and absence of $^{13}$C-DOC showed our mesocosm-based experimental design as sufficiently robust to investigate the utilization of $^{13}$C-DOC by sediment microbial communities. After 48 hours of incubation, PLFA-SIP identified heterotrophic $\alpha$, $\beta$, and $\gamma$- proteobacteria and facultative anaerobic bacteria as the organisms primarily responsible for humic DOC consumption in streams and heterotrophic microeucaryotes as their predators. The evidence presented in this study shows a complex relationship between microbial community structure, environmental heterogeneity and utilization of humic DOC, indicating that humic DOC quality and quantity along with other hydro-ecological variables should be considered among the important factors that structure benthic microbial communities in lotic ecosystems.
DEDICATION

I dedicate this piece of work to my family: Taiwo, Susan, Daniella and David for they are there for me every single day.
## LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Anteiso</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>br</td>
<td>Branched</td>
</tr>
<tr>
<td>$^{12}\text{C}$</td>
<td>Carbon with a mass of 12</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>Carbon with a mass of 13</td>
</tr>
<tr>
<td>$\text{C}_{18}$</td>
<td>18 carbon chain</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>cy</td>
<td>Cyclo</td>
</tr>
<tr>
<td>$df$</td>
<td>Degrees of freedom; number of values free to vary after certain restrictions have been placed on the data</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
</tr>
<tr>
<td>FAMEs</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>FHC</td>
<td>High flood pulse count</td>
</tr>
<tr>
<td>FIG</td>
<td>Figure</td>
</tr>
<tr>
<td>FLC</td>
<td>Low flood pulse count</td>
</tr>
<tr>
<td>ffw</td>
<td>Fresh wet weight</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
</tbody>
</table>
gdw  Gram dry weight

GC/C/IRMS  Gas chromatography-combustion-isotope ratio mass spectrometry

GF/F  Glass fiber filters

h  Hour

ha  Hectare

iso  Iso

IHV  Indicators of hydrological variation

kDa  Kilodalton

km  Kilometer

L  Liter

ln  Natural log

m  Meter

m²  Meter squared

m³  Meter cubed

M  Mean: the sum of a set of measurements divided by the number of measurements in the set

MBI  Base flow index

MDF  Mean daily flows

MQ50  Median daily flow

MVD  Variability in daily

mg  Milligram

min  Minute

mL  Milliliter

mm  Millimeter
n  Number of sample size

$^{14}$N  Nitrogen with a mass of 14

ng  Nanogram

nmol  Nanomole

NIST  National Institute of Standards and Technology

p  Probability associated with the occurrence under the null hypothesis of a value as extreme as or more extreme than the observed value

pH  Concentration of hydrogen ions

PCA  Principle component analysis

PLFA  Phospholipid fatty acids

PLP  Phospholipid phosphate

ppm  Parts per million

r  Pearson product-moment correlation

s  Second

$t$  Computed value of $t$ test

v  Volume

v/v/v  Volume to volume

V-PDB  Vienna Pee Dee Belemnite standard

<  Less than

>  Greater than

=  Equal to

$\alpha$  Alpha

$\beta$  Beta

$\gamma$  Gamma
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>ω</td>
<td>Omega</td>
</tr>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>‰</td>
<td>Per mille</td>
</tr>
<tr>
<td>ºC</td>
<td>Temperature in Celsius</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

As I hit on the keyboard of my laptop while writing this section of my dissertation, I realize that I am within a few days of completing my doctoral studies, after a long tiring process that have presented some challenges. I got to this point because of so many gracious people that helped nudged me ahead and made valiant efforts to keep me prepared for the next challenges.

My best conceivable advisor: Bob Findlay:
Words cannot express my heartfelt gratitude, appreciation and thanks for all the support, guidance and time you provided for this research project and my graduate training; right from the day you picked me up at the Atlanta International Airport to the completion of this dissertation. You walked me though the dark moments of my academic birth pangs and believed in me; that worked into something good. Thank you.

My Dissertation Committee- Drs. Ward, Olson, Mortazavi and Andrus:
Your dedication, guidance, consideration and insightful suggestions are greatly appreciated. Thank you for your constructive comments every step of the way and all the little ‘extra push’ so that I can become better. Thank you.

Department of Biological Sciences and Graduate School:
I express my thanks to the Department of Biological Sciences for the teaching assistantship that provided much needed stipend and exposure to classroom settings. I am grateful to the Graduate school for Dean’s Discretionary Scholarship for the Fall 2009 semester towards the successful completion of my degree. Thank you.
Collaborating institute and teams:

Stroud Water Research Centre, Avondale, PA and Stable Isotope Biogeochemistry Lab, Michigan State University. Thanks to all the people who stayed with me through all the challenges and pitfalls of experimental work: setting up mesocosms, collecting sediment samples, putting me through the GC/C/IRMS, including their support in several inches of snow in Michigan and hiking the Catskill Mountain in New York: Lous Kaplan, Robert Sherman, Michael Gentile, Peggy Ostrom and Hasand Ghandi. Thank you.

My lab mates and colleagues:

Janna Brown, Prarthana Ghosh, Jen Mosher, Thomas Branan, Joshua Mays, Edwina Clarke, Brian Shirey, Michael Kendrick and Elise Chapman, for technical and logistic supports, and taking time out of no time to share information and knowledge at various levels. Thank you.

My awesome family:

Special thanks to my best friend and loving wife; Taiwo and beautiful kids, Susan, Daniella and David. With your love and support despite my little stipend and my ‘absenteeism’ at home, you have made this adventure successful. We made it together! Finally, to my families and friends at ‘home,’ thousands of miles away for asking me to finish my studies in time because I need to start making money. Thank you for those reminders and your understanding all the way.

To Him:

‘…But you are not dead: you lives and abides forever,
For in you we live and move and have our being’

*Epimenides of Crete*
# TABLE OF CONTENTS

ABSTRACT ............................................................................................................. ii

DEDICATION ........................................................................................................... iv

LIST OF ABBREVIATIONS AND SYMBOLS .................................................... v

ACKNOWLEDGMENTS ............................................................................................... ix

LIST OF TABLES ........................................................................................................ xii

LIST OF FIGURES ..................................................................................................... xiii

CHAPTER 1: GENERAL INTRODUCTION ............................................................. 1

CHAPTER 2: SPATIAL PATTERNS OF MICROBIAL SIGNATURE BIOMARKERS IN STREAM NETWORKS ................. 12

CHAPTER 3: SEASONALITY IN A STREAMBED MICROBIAL COMMUNITY: VARIATION IN THE ISOTOPIC COMPOSITION OF LIPID BIOMARKERS ............................................. 52

CHAPTER 4: ELUCIDATING THE BACTERIA RESPONSIBLE FOR UTILIZATION OF DISSOLVED ORGANIC MATTER IN A THIRD-ORDER STREAM ......................................................... 96

CHAPTER 5: OVERALL CONCLUSIONS ............................................................... 134

REFERENCES ........................................................................................................... 140

APPENDIX .................................................................................................................. 147
**LIST OF TABLES**

**TABLE 1.1** Personal contribution of POA to the NSF funded project and publications.................................................................................................................................................................9

**TABLE 2.1** Microbial biomass, water chemistry and sediment organic content of White Clay Creek and Neversink watersheds...........................................................................................................49

**TABLE 2.2** Nested ANOVA to test the effects of watershed, streams within watershed, and stations within streams on microbial biomass..............................................................50

**TABLE 2.3** Multiple regression analysis (best subsets) for natural log biomass as a function of various physical and chemical stream parameters.................................51

**TABLE 3.1** Seasonal variations in total sedimentary microbial biomass, bacterial abundance, and physico-chemical parameters of White Clay Creek...............91

**TABLE 3.2** Pearson correlation coefficient matrices between selected hydrological indices and measured environmental variables.........................................................92

**TABLE 3.3** Multiple regression analysis (best subsets) for natural log biomass as a function of stream physico-chemical and hydrological indices...............................93

**TABLE 3.4** Multiple regression analysis (best subsets) for microbial community (PC1) as a function of stream physico-chemical and hydrological indices.......94

**TABLE 3.5** Annual variations in stable carbon isotope signatures for selected common fatty acids from White Clay Creek sediments. Values shown are mean (±SD) from all sampling sites and months.........................95

**TABLE 4.1** Experimental design of $^{13}$C-DOM uptake experiments.................................................................131

**TABLE 4.2** Microbial PLFA $\delta^{13}$C values (%c; mean ± SD) from 6 mesocosm experiments determined using DB-1 and DB-23 chromatographic columns..........................132

**TABLE 4.3** Phylogenetic affiliation of bacterial fatty acids functional groups extracted from White Clay Creek sediment.................................................................133
LIST OF FIGURES

FIG 1.1  Mesocosm setup for $^{13}$C leachate uptake measurement, including streamwater-fed bioreactors/ mesocosm chambers containing sediments and water jackets. One chamber without $^{13}$C leachate amendment served as experimental control

FIG 1.2  Close up view of mesocosm chamber showing top surface of a galvanized sediment box contiguous with the front ramps of the Venturi flumes and associated recirculating pipes system

FIG 2.1  Sampling scheme used to examine microbial biomass and community structure across multiple spatial scales in two watersheds. Sampling within the Neversink watershed consists of four 1$^{st}$ order streams; Biscuit Brook and Pigeon Creek tributaries (Biscuit Brook Tributary A and B [BBTA, BBTB], Pigeon Creek Tributary A and B [PPTA, PPTB], 1a, 1b, 1c, 1d, respectively), two 3$^{rd}$ order streams (Biscuit Brook [BBR] and Pigeon Creek [PBR], 3a, 3b, respectively) and one 5$^{th}$ order stream (Neversink River [NRC]). Sampling within the White Clay Creek watershed consists of four 1$^{st}$ order streams (Ledyards Spring Branch [LSB], Water Cress Spring [WTR], Dirty Dog Spring [DDS] and Walton Spring Branch [WSB], 1e, 1f, 1g, 1h, respectively), two 2$^{nd}$ order streams (East and West Branch White Clay Creek [WCWE, WCCW], 2a, 2b, respectively) and one 3$^{rd}$ order stream (White Clay Creek [WCC]). Sketches of watersheds are not drawn to scale. Each eclipse represents a reach, which contained 3 stations, each of which was sampled times

FIG 2.2  Variation in sediment (a) percent carbon, (b) percent nitrogen and (c) C:N ratio by stream, order (1$^{st}$ to 3$^{rd}$/5$^{th}$ order from left to right) and watershed. Vertical bars denote 0.95 confidence intervals. Streams not connected by a horizontal line are significantly different (p = 0.05, Tukey’s Wholly Significant Difference)

FIG 2.3  Microbial biomass (mean ± SD) of White Clay Creek and Neversink watershed sediments at three spatial scales: a; watershed, b; stream and c; station. Stream order (or average order for watershed values) are indicated as: black = 1$^{st}$ order, dark gray = 2$^{nd}$ order, light gray = 3$^{rd}$ order, open = 5$^{th}$ order
FIG 2.4  Path diagrams describing the structure of the relationship between sediment microbial biomass and % Carbon, % water content, C:N ratio and sediment surface area. Single-headed arrows indicate casual paths; numbers on arrows are path coefficients (standardized regression coefficients) indicating the relative strength of each path leading to a given response variable. Double-headed arrows represent the correlations among the predictor variables. Arrows connecting environmental variables to the independent variable (microbial biomass) indicate direct effects, while environmental variables linked to the independent variable via other environmental variable constitute indirect effects. Path coefficients calculated by SAS Structural Equation Modeling for JMP 10. *= P <0.01, **=P <0.001……………………………………… …………………… …45

FIG 2.5  Principle Component Analysis of stream sedimentary microbial community structure of White Clay Creek (open circle) and Neversink (open square) watersheds. The percent variation explained by each axis is indicated on the respective component axis. Identified fatty acids had component loadings of >|0.5| with strong influence on the pattern of variation among samples along the respective component axes. Site abbreviations are as described in the legend to Fig.1…………………………………………………………… ……46

FIG 2.6  Relationship between Principle Component Analysis factor 1 score and the calculated percentage that microeukaryotes contribute to total microbial biomass for all stream samples……………………………………………………………………… 86

FIG 2.7  Spatial variation in sedimentary bacterial community composition in WCC and NRC watersheds by PLFA analysis after removal of fatty acids assigned a priori to the functional group microeukaryotes and those known to be common to both bacteria and microeukaryotes from the PLFA profiles. Symbols- WCC (circle), NSR (square). Site abbreviations are as described in the legend to Fig.1……………………………………………………………………… 87

FIG 3.1  Mean daily discharge at United States Geological Survey (USGS) gauging station of the study stream during the study period from November 2009 to October 2010. Arrow represents sampling date of streambed sediment samples……………………………………………………………………… 86

FIG 3.2  PCA of benthic microbial community structure determined by PLFA from the White Clay Creek seasonal sampling site. Scores are plotted by months: February, F; March, Ma; April, Ap; May, My; June, Ju; July, Jy; August, Au; September, S; October, O; December, D. Scales indicate the degree of difference among samples and influential fatty acids (factor loadings > |0.5|) are shown along each axis. Symbols indicate mean PC scores (n=9, except Nov. and Dec. where n =3), error bars = ±S.D……………………………………………………………………… 87

FIG 3.3  Relationship between PCA factor 1 score and the calculated percentage that microeukaryotes contribute to total microbial biomass for all samples……………………………88
FIG 3.4 Seasonal variability in sedimentary TOC and selected $\delta^{13}$C PLFAs with component loadings >0.5 that exerted strong influence on the pattern of variation among samples along the PC 1 (Fig 5). Bars represent standard deviation.............89

FIG 3.5 PCA of all quantified $\delta^{13}$C of PLFAs of WCC benthic microbial community. Scores are plotted by months: February, FE; March, MA; April, AP; May, MY; June, JU; July, JY; September, SE; October, OC; December, DE. Influential fatty acids (factor loadings > |0.5|) are shown along each axis. # summed feature includes 16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t; *summed feature includes 18:2ω6, 18:3ω3, 18:1ω9, 18:1ω7c, 18:1ω5…………………………………………90

FIG 4.1 Changes in a) microbial biomass, b) percent prokaryotes and c) community structure summarized by PCA axis 1, among treatments and sampling dates for all experiments. Values are mean differences ± SD, (n = 6). T0-TM= Differences attributed to mesocosm effect, TM-$^{13}$C= Differences attributed to the effects of $^{13}$C-labeled DOM…………………………………………130
CHAPTER 1
GENERAL INTRODUCTION

Microbes are important players in lotic ecosystems and are responsible for several biogeochemical transformations, including liberation of essential nutrients via detrital decomposition and dissolved organic matter uptake, degradation, and mineralization (Kaplan and Newbold 1993; Pusch et al. 1998; Fischer and Pusch 2001; Tank et al. 2010). They have small size, ubiquitous distribution, high surface to volume ratio, short generation intervals, high metabolic diversity and the highest documented intraspecific genetic diversity of any type of organism (McArthur et al. 1988; Morehead et al. 1996). Microbial processing of terrestrial particulate organic matter (POM), dissolved organic matter (DOM) and nutrients within the lotic ecosystems control the material flux that influence higher trophic levels (Dobbs and Guckert 1988; Hart 1992; Poff and Ward 1992; Pusch et al. 1998; Cotner and Biddanda 2002). The utilization of DOM in streams by heterotrophic microbial community controls important lotic ecosystem processes and supports productivity at higher trophic levels. It is, therefore, important not only to describe microbial community structure and function, but also, to identify biological processes and environmental variables that influence their assemblages both temporally and spatially.

DOM is the largest active pool of carbon in lotic ecosystems and is continuously supplied to the system from both allochthonous (terrestrial) and autochthonous (aquatic) sources (Peduzzi et al. 2008). Mounting evidence has shown that it plays a significant role in aquatic ecosystems
as carbon and energy sources for the microbial food web (Peduzzi et al. 2008; Wiegner et al. 2009; Wong and Williams 2010), and its flux from streams and rivers often dominates organic loading to estuaries (Amon and Benner 1996). In addition, due to its dynamic role in the interaction between hydrosphere and biogeosphere, DOM is now seen as an important driver of ecosystem functions in freshwater environments and a major component in global carbon cycling and climate change (Amon and Benner 1996; Batin et al. 2008; Besemer et al. 2009). The interactions between both the quantity and quality of DOM and stream microorganisms are important to several key ecosystem functions. Variations in the quality and quantity of DOM can exert pronounced influence on microbial communities altering characteristic such as biomass, enzymatic activities, and community structure (Bourguet et al. 2009; Freese et al. 2010, Mosher and Findlay 2011). For example, experimental manipulations of organic matter concentration and composition have shown marked changes in bacterial metabolic activities (Smith et al. 1995; see Findlay and Sinsabaugh 1999 and reviews therein). Also, microbial processes directly influence qualitative and quantitative transformations of DOM in the environment (Bourguet et al. 2009). Thus, the fate of DOM is intimately associated with microorganisms that are responsible for carrying out a wide range of processes that are fundamental to ecosystem success. However, research efforts to understand DOM utilization through microbial processes have been complicated by the chemical heterogeneity of the DOM pool and a lack of methods for measuring \textit{in situ} microbial activities (Kaplan et al. 2008; Bourguet et al. 2009).

In the past, our knowledge has been on bulk microbial processes, generally treating microbial community as a “black box” (Cottrell and Kirchman 2000; Foreman and Covert 2003). Studies linking bacterial community structure with functions, such as DOM turnover, are few and mostly focus on the microbial processing of tracers that are not reflective of natural stream
DOM. Early attempts include NaH$_{13}$CO$_3$ additions in lakes (Kritzberg et al. 2004; Pace et al. 2004) and $^{13}$C-enriched sodium acetate additions in streams (Hall and Meyer 1998; Johnson and Tank 2009; but see Kaplan et al. 2008). However, with the development of new techniques and substrate (e.g., leachate from composted $^{13}$C-labelled tulip poplar tree-tissues; Wiegner et al. 2005a), we can examine what components of DOM are susceptible to degradation and understand factors affecting taxon-specificity in utilization ability and improve models of carbon and energy transformation in aquatic habitats. Thus, we can begin to elucidate which microbial functional group utilizes a given DOM constituent and if humic DOM supports a significant portion of stream ecosystem metabolism.

Unlike DOM, the scaling of uptake lengths of nutrients with stream size has been widely documented (Newbold et al. 1981; Peterson et al. 2001; Hall et al. 2002; Alexander et al. 2007; Tank et al. 2008; but see Kaplan et al. 2008). For example, uptake lengths are shorter in headwater streams, whereas streams with greater depth and velocity (i.e., 4$^{th}$ order streams and above) will have longer uptake lengths (Hall et al. 2002). Our understanding of bacterial functional group utilization of DOM constituents will not be complete without considering the spatial scale at which these ecological processes occur. Fortunately, the hierarchical nature of stream networks, in which a series of successively smaller geomorphic units are nested within each other (Lowe et al. 2006), makes it a prime candidate to test the application of scaling rules to DOM uptake across stream orders. In effect, riffles/pools are nested within stream reaches, which are nested within streams, which are nested within watersheds. Both theoretical models and experimental evidence have demonstrated that the scale at which ecological processes occur has an effect on microbial community and diversity (Durrett and Levin 1997; Kerr et al. 2002). However, studies on systematic assessments of variability in biological aspect of DOM and
microbial metabolism across multiple spatial scales are rare. Such an approach, when coupled with measurements of bio-physiochemical variables, could inform how the controls of microbial utilization of DOM occur across temporal and spatial scales.

The stable isotope ratios of carbon (measured on isotope ratio mass spectrometers) have the potential to serve as tracers for sources, flow paths and transformations of dissolved and particulate organic carbon in lentic and lotic ecosystems (e.g., Hall 1995; Hall and Meyer 1998; Cole et al. 2002). Measurements of δ\(^{13}\)C have been used to investigate DOM dynamics in a wide variety of streams and laboratory mesocosms (Hall and Meyer 1998; Cole et al. 2002; Wiegner et al. 2005b). An exciting innovation in isotope ratio analysis is the development of gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). This improved technique can be used to link microorganisms in environmental samples to utilization of particular growth substrates (Dumont and Murrell 2005). Presently, lipids, amino acids and nucleic acids have been used as the biomarker molecules and for compound-specific stable isotope analysis (Boschker et al. 1998; Radajewski et al. 2000). Phospholipid fatty acid analysis (PLFA) and lipid profiling are well-established techniques for the identification of microorganisms and characterization of microbial communities (White et al. 1994; Findlay et al. 1989; 2004). PLFA analysis for assessing the activity of microbial communities in the environment is greatly augmented by the use of \(^{13}\)C-labelled substrates in conjunction with GC/C/IRMS (Boschker 2004; Evershed et al. 2006). This approach has proved successful for linking specific populations within complex microbial consortia with substrate utilization through \(^{13}\)C enrichment of PLFA biomarkers in various environments and situations (Boschker et al. 1998). Thus, combining \(^{13}\)C-DOM produced using the technique developed by Wiegner et al. (2005a) with GC/C/IRMS should provide insights and important information about the dynamics
of DOM in aquatic ecosystems and the heterotrophic microorganisms responsible for its uptake and utilization. The biological lability of DOM, including terrestrial derived humic substances, and consequently, its importance to bacterial metabolism, underpins the focus of my dissertation. This study has employed compound-specific stable isotope and PLFA techniques to elucidate the bacteria responsible for utilization of humic DOM in streams and to assess overall variability in microbial biomass and community structure temporally and across multiple spatial scales in stream networks.

The first question addressed is “how similar are stream microbial communities across multiple spatial scales within and among stream networks” (Chapter 2). Most studies have shown horizontal variations in microbial abundance, distribution and diversity within a stream continuum or among streams (Battin et al. 2001; Oda et al. 2003; Crump et al. 2004; Hughes-Martiny et al. 2006; Fierer et al. 2007), but little attention has been paid to the significance of multiple spatial scales in stream microbial ecology studies. This research effort investigates microbial biomass and community structure from streambed sediments in 14 streams within two forested watersheds across four spatial scales: among individual sediment cores; within reaches within stream; among streams within watershed and between watersheds. In addition, factors or set of factors that control the structure of microbial communities in these systems were investigated. Although some studies have suggested environmental variables that influence microbial communities in several habitats (Battin et al. 2001; Gao et al. 2005; Fierer et al. 2007), how environmental heterogeneity structures microbial community composition and distribution in streambed is not fully understood.
Chapter 3 examines seasonal variations in stable carbon isotope signatures of individual microbial fatty acids of White Clay Creek, a 3rd order piedmont stream in southeastern Pennsylvania. To the best of our knowledge, there have been no reports describing seasonal variation in compound specific carbon isotope signatures of individual PLFA in relation to changes in microbial community structure in stream sediments. Understanding the seasonal variation of isotopic signatures of specific microbial biomarkers will yield valuable insights into dynamics of the carbon isotopic composition of the biological and sedimentary substrates in streams. Unlike studies on seasonal variations in assemblages of stream fishes and invertebrates, (e.g., Bott and Borchardt 1999; Pires et al. 1999; Cowell et al. 2004; Cleven 2004; Taylor et al. 1996; Hatzenbeler et al. 2000; Davey and Kelly 2007), studies that have investigated the structure and seasonal dynamics of sedimentary microbial communities are limited in number (Kaplan and Bott 1989; Smoot and Findlay 2001; Battin et al. 2001; Sutton and Findlay 2003). Thus, more detailed information on seasonal patterns in benthic microbial community composition and associated carbon isotope signatures are needed to fully understand the use of stable isotope probing in addressing the question of DOM use by stream microbiota.

Chapter 4 elucidates which heterotrophic benthic microbes within streams actively utilize DOM and ultimately control the material flux that influences higher trophic levels. Although viewed as biologically more recalcitrant and perhaps less energy yielding than monomers (Amon and Benner 1994), evidence has shown that a portion of the humic substances is biologically degradable (Moran and Hodson 1990; Carlsson et al. 1999). The research efforts in this chapter employ bioreactors (Figure 1.1a and b) fitted with undisturbed (as much as possible) sediments as a laboratory tool to study the incorporation of synthesized tree tissue leachate (Wiegner et al. 2005a) into microbial PLFA biomarkers. The compound-specific $^{13}$C analyses of individual
PLFAs will allow us to identify the microbial functional group(s) responsible for the uptake of humic substances in streamwater. Leachate used in this study was synthesized from tulip poplar seedlings that were earlier grown with $^{13}\text{CO}_2$ at the National Phytotron located at Duke University, Durham, North Carolina, USA (Wiegner et al. 2005a).

Chapter 5 provides a summary of the major results and conclusions from the aforementioned chapters and directs attention to their implications in terms of future research efforts in microbial ecology. This interdisciplinary project contributes to the understanding of the utilization of labile and semi-labile DOM in streams and provides insight into the efficacy of mesocosms as tools within microbial ecology. However, my dissertation extends the study of microbial utilization of labile and semi-labile DOM by including environmental influences that influence temporal and spatial distributions of microbial community in streams, producing a better understanding of the importance of headwaters to river networks and important implications for the protection of forested headwater streams.
CONTRIBUTION TO PUBLICATION AND MANUSCRIPTS

My doctoral dissertation was a part of a larger NSF-funded collaborative project to address the application of scaling rules to energy flow in stream ecosystems; however, my focus centered on investigating spatio-temporal variations in microbial community in stream networks and elucidating the bacteria responsible for utilization of dissolved organic matter in streams. Consequently, my PhD dissertation was interdisciplinary and completed in close collaboration with Lou Kaplan (biogeochemistry) at Stroud Water Research Centre, Avondale, PA, where mesocosm experiments were conducted; Peggy Ostrom (geochemistry) at Michigan State University, East Lansing, MI, where I carried out compound specific stable isotope analysis; and Robert Findlay (microbial and ecosystem ecology) at University of Alabama, Tuscaloosa, AL, where I had my graduate training.

The work presented here is based on 3 manuscripts prepared by P.O. Akinwole for submission to peer-reviewed journals; the close interdisciplinary cooperation will result in a number of co-authorships. These are:


- **Akinwole P.O.**, L.A. Kaplan and R. H. Findlay. (in prep). Seasonal variations in the carbon isotopic composition of lipid biomarker compounds and structure of a streambed microbial community. To be submitted to *Microbial Ecology*

Personal contributions of P.O. Akinwole to the interdisciplinary project and included manuscripts are shown in Table 1.

Table 1.1  Personal contribution of POA to the NSF funded project and publications.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Chapter 2</th>
<th>Chapter 3</th>
<th>Chapter 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental design</td>
<td>xxx</td>
<td>xx</td>
<td>x</td>
</tr>
<tr>
<td>Experimental/Field work</td>
<td>xxx</td>
<td>x</td>
<td>xx</td>
</tr>
<tr>
<td>Laboratory analyses</td>
<td>xxx</td>
<td>xxx</td>
<td>xxx</td>
</tr>
<tr>
<td>Data analysis</td>
<td>xxx</td>
<td>xxx</td>
<td>xxx</td>
</tr>
<tr>
<td>Manuscript writing</td>
<td>xxx</td>
<td>xxx</td>
<td>xxx</td>
</tr>
</tbody>
</table>

xxx = major contribution; xx = moderate contribution; x = minimum contribution

Chapter 2: Spatial pattern of microbial signature biomarkers in stream networks

Chapter 3: Seasonality in a streambed microbial community: variation in the isotopic composition of lipid biomarker

Chapter 4: Elucidating the bacteria responsible for utilization of dissolved organic matter in a third-order stream
Fig. 1.1 Mesocosm setup for $^{13}$C leachate uptake measurement, including streamwater-fed bioreactors/ mesocosm chambers containing sediments and water jackets. One chamber without $^{13}$C leachate amendment served as the experimental control.
Fig. 1.2. Close up view of mesocosm chamber showing top surface of a galvanized sediment box contiguous with the front ramps of the Venturi flumes and associated recirculating pipes system.
CHAPTER 2

SPATIAL PATTERNS OF MICROBIAL SIGNATURE BIOMARKERS IN STREAM NETWORKS

ABSTRACT

The large-scale spatial patterns of microbial community structure and diversity are largely unknown compared to those of macro fauna and flora. We investigated these patterns in stream sediments from two watersheds; the Neversink River watershed (NY; 1st, 3rd and 5th order streams sampled) and the White Clay Creek watershed (PA; 1st through 3rd order streams sampled). Microbial biomass and community structure were estimated by phospholipid phosphate and phospholipid fatty acids (PLFA) analyses, respectively. Multivariate analysis showed that C:N ratio, percent carbon, sediment surface area and percent water content explained 68% of the variations in total microbial biomass. Overall, the variability of microbial biomass within streams was low compared to the variability among streams and between watersheds. Principal component analysis of PLFA profiles showed that microbial community structure displayed a distinct watershed-level biogeography, as well as variation along a stream order gradient. This study indicates a non-random distribution of microbial communities and that environmental heterogeneity and geographical distance can influence microbial distribution.

Key words: benthic microbial community, microbial biomass, Neversink and White Clay Creek watersheds, multiple spatial scales, phospholipid fatty acids
INTRODUCTION

Microbial taxa are the most biologically diverse and ubiquitous taxa on earth and their metabolic activities largely control biogeochemical cycling and ecosystem processing (Curtis and Sloan 2004; Tringe et al. 2005; Tank et al. 2010). In stream ecosystems, benthic microbial communities mediate many of the biochemical transformations, including degradation and transformation of recalcitrant chemical compounds into biomass or inorganic components, exerting significant control over the mineralization and downstream exportation of terrestrially-derived dissolved organic matter (DOM) (Kaplan and Newbold 1993; Pusch et al. 1998; Fischer and Pusch 2001; Tank et al. 2010). In addition, microbial processing of terrestrial-DOM and nutrients within the streambed sediments is essential to material flux to higher trophic levels (Hart 1992; Poff and Ward 1992; Pusch et al. 1998; Hall and Meyer 1998). Consequently, microbes are best described as life’s engines driving biogeochemical processes in streams, as well as on earth (Falkowski et al. 2008).

There has been limited progress in our understanding of how microbial diversity changes across spatial gradients and comparable research on the microbial biogeographical patterns have lagged behind research on plant and animal communities (Fierer and Ladau 2012). Attempts to investigate microbial biogeography in stream sediments have shown the emergence of clear biome-level patterns in streambed microbial communities (Findlay et al. 2008). Gao et al. (2005) compared benthic bacterial community structure among nine streams across the southeastern and midwestern United States and observed differences attributed to variations in chemical characteristics of the habitats, rather than a pattern driven by spatial gradients. Other studies have
shown that microorganisms vary in abundance, distribution and diversity over various habitats and that microbial composition across landscapes is nonrandom (Øvreås et al. 1997; Cho and Tiedje 2000; Battin et al. 2001; Oda et al. 2003; Crump et al. 2004; Hughes-Martiny et al. 2006; Fierer et al. 2007)

How microbial diversity across spatial scales is related to the physical, chemical and biotic variables of ecosystems is a fundamental question in microbial ecology. Fierer et al. (2007) found that a single variable, streamwater pH, could predict much of the variability in bacterial communities inhabiting fine benthic organic matter across the Hubbard Brook watershed. Changes in quantities and qualities of carbon availability may also alter microbial community structure in predictable ways (Fierer et al. 2007; Nemergut et al. 2010). Other studies found that sediment chlorophyll a (Battin et al. 2001; Gao et al. 2005), dissolved organic carbon and nitrate concentrations (Gao et al. 2005), stream order and current regime (Molloy 1992) were other environmental variables influencing the structure of microbial communities.

Although our understanding of microbial biogeography continues to expand, there is a paucity of information on the spatial distribution of microbial communities attached to streambed substrata of low-order streams and on the factors that control their distributions (Leff 1994). Particularly, low-order streams serve as important links between terrestrial and larger aquatic systems (Hullar et al. 2006). Understanding the ecological coherence (Philippot et al. 2011) of benthic microbial community in headwater streams may have important implications for ecological linkages between aquatic and terrestrial systems. Stream networks are inherently hierarchical in nature with a series of successively smaller geomorphic units nested within each other (reaches are nested within streams and streams within watersheds) (Tiegs et al. 2009). Characterization of stream networks and the variation in microbial communities across these
networks require a sampling regime that adequately captures this complexity. This study examined microbial biomass and community structure from streambed sediments in forested streams within two distinct watersheds. We used a nested sampling design and sampled at four spatial scales: within a station (individual sediment cores separated by <1m); among stations within a stream reach (separated by >1m but ≤50m); among streams within watershed (separated by >50m but ≤10km) and between watersheds (separated by >350km). Our study was designed to investigate how similar stream microbial communities were across multiple spatial scales within and among stream networks. We hypothesized that increasing spatial scale of fluvial geomorphology units, from reaches to watersheds, increases variability in microbial communities at each hierarchical level. We sampled streambed sediments from 1st, 2nd and 3rd - order streams in White Clay Creek stream network (Avondale, PA, USA) and from 1st, 3rd and 5th- order streams in the Neversink stream network (Claryville, NY, USA). We used phospholipid-based techniques to characterize the microbial biomass and community structure of sediments from these streams. Our data were subjected to multivariate statistical analyses to compare the patterns of microbial community structure within and between stream networks.
METHODS

Study sites and experimental design

Study streams were located within two stream networks: the 3rd order, 7.3 km² White Clay Creek (WCC) watershed in the southern Pennsylvania Piedmont, and the 5th order, 171 km² Neversink River (NSR) watershed within the Catskill Mountains of New York. The WCC watershed is located within the Piedmont Province of southeastern Pennsylvania and predominant land uses are agricultural (52%), hayed/grazed fields (22%) and wooded lands (23%) (Wiegner et al. 2005; Newbold et al. 1997). Streamflow and streamwater chemistry have been monitored since the 1970s with mean annual stream flow, stream water temperature, and local precipitation of 115 L/s, 10.6°C, and 105 cm, respectively. Streambed sediments consist of clay-, silt-, and sand-sized particles in pools and runs, with gneiss- and schist-derived gravel and cobble in riffles. The dominant tree species reported are beech (*Fagus grandifolia*), red oak (*Quercus rubra*), black oak (*Quercus velutina*) and tulip poplar (*Liriodendron tulipifera*). Detailed description is given in Newbold et al. (1997). The Neversink watershed is contained within a mountainous region in northeast New York State and elevation ranges from 480 m to 1280 m. The hill slopes are steep with several deeply incised headwater channels and the soils in the Catskills region are predominantly acidic inceptisols (Lawrence et al., 2001). Streambed sediments consist of clay-, silt-, and sand-sized particles and shale-, siltstone-, sandstone- and conglomerate-derived gravel and cobble in riffles. The watershed is sparsely populated and 95% forested, primarily of mixed northern hardwood species dominated by American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*) and yellow birch (*Betula alleghaniensis*). Balsam fir (*Abies balsamea*) is common
above 1,000-m elevation, and hemlock stands grow in a few areas that have poorly drained soils (Lawrence et al. 2001; Lovett et al. 2002).

We used a hierarchical design to evaluate spatial patterns of microbial biomass and community structure along a stream order gradient and across four spatial scales, where stream order refers to Strahler’s (1957) modification of Horton’s (1945) classification system (headwater streams with no tributaries are 1st order, two first order streams join to form a 2nd order stream, when two 2nd order streams combine, they form a 3rd order stream and so on). Our nested sampling design consisted of four spatial scales: 1) > 350km - distance between the watersheds, 2) 50m-10km - distance between streams within a watershed, 3) 1-50m - distance between sampling stations within a stream reach, and 4) <1m – the distance between replicate cores within a sampling station (Fig.1). In White Clay Creek stream network, we sampled 3rd order WCC adjacent to the Stroud Water Research Center in Avondale, Pennsylvania, two 2nd order streams; White Clay Creek West (WCCW) and White Clay Creek East (WCCE) and four 1st order streams; Ledyards Spring Branch (LSB), Water Cress Spring (WTR), Dirty Dog Spring (DDS) and Walton Spring Branch (WSB). Two 1st order streams flowed into each 2nd order stream (LSB and WRT into WCCW, DDS and WSB into WCCE). In Neversink stream network, we sampled the 5th order Neversink River (NRC), two 3rd order streams; Biscuit Brook (BBR) and Pigeon Creek (PBR) and four 1st order streams; Biscuit Brook Tributary A (BBTA), Biscuit Brook Tributary B (BBTB), Pigeon Creek Tributary A (PBTA) and Pigeon Creek Tributary B (PBTB). Pigeon Creek, Biscuit Brook and their tributaries are located within the Frost Valley, Claryville, Ulster County, NY. Within each stream, three stations within a reach (downstream, midstream and upstream) were established and triplicate sediment samples collected at each station. In summary, the design consists of 2 stream networks, 7 streams per stream network, 3 stations per stream and
3 replicate sediment samples per station, corresponding to a total of 126 sediment samples. Within the watershed sampled, both rivers were unregulated. All streams within a watershed were sampled in the same week, and both watersheds were sampled within a 2-week period in July and August 2010 to avoid seasonal differences.

**Sampling procedures**

Samples were delimited with a 100mm diameter Plexiglas ring that was inserted 2cm deep into the streambed (75mm diameter ring was used for 1st order streams whenever streambeds were dominated by large rocks, cobbles and stones). Plexiglas plates were slipped under and over the ring to effectively trap the sediments and allow them to be lifted from the streams without disturbance. Sediments in the top 2mm within the ring were transferred with a clean spatula to pre-labeled Whirl-Pak sampling bags and stored on ice prior to subsampling. Within six hours of sampling, sediments were transferred to a clean plastic weigh boat, thoroughly homogenized and subsampled for phospholipid, surface area and elemental analyses. Subsamples for phospholipid and elemental analyses were frozen and shipped to the appropriate laboratory for analysis. Conductivity and water temperature readings were measured with a YSI model 32 conductance meter.

**Phospholipid analysis**

Microbial biomass and community structure were determined using phospholipid phosphate (PLP) and phospholipid fatty acid (PLFA) analyses following the methods of Findlay (2004). Briefly, cellular lipids were extracted from the frozen sediment samples by dichloromethane/methanol/water extraction and partitioned into aqueous and organic fractions. The organic fraction containing the lipids was subsampled for PLP analysis (Findlay et al. 1989). PLFA were fractionated from the remaining lipids by silica gel solid phase extraction.
chromatography using chloroform (neutral lipids), acetone (glycolipids) and a solution of chloroform:methanol:DI water (5:5:1, v:v:v;) as successive eluents. PLFAs were converted into their respective methyl esters by base methanolysis and purified by octadecyl bonded silica gel (C18) reverse-phase column chromatography. Purified fatty acid methyl esters (FAMEs) were identified and quantified using gas chromatography. The FAMEs were analyzed by gas chromatography in an Agilent gas chromatograph equipped with an automatic sampler, a 60 m x 0.25 mm non-polar DB-1 column and a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 2.3 ml/min. The initial temperature was 80º C followed by a temperature rise of 4 ºC/min to 250 ºC which was then held at this temperature for 10 min. FAME identification was based on relative retention times, coelution with standards, and mass spectral analysis. The FAME nomenclature used followed Findlay and Dobbs (1993). Using polyenoic fatty acids as indicators of microeukaryotes, total microbial biomass was partitioned between prokaryotic and microeukaryotic organisms and the results presented as percentages (Findlay and Dobbs 1993).

Elemental Analysis

The frozen subsamples for elemental analysis were freeze-dried, finely ground, weighed (about 1.5 - 2g) and inorganic carbonate removed by exposure to gaseous HCl. Approximately 35 mg of sediment was analyzed on a Costech 4010 elemental analyzer for percent carbon and nitrogen, and atomic carbon to nitrogen ratio (C:N). Stable isotope ratios ($\delta^{13}$C, $\delta^{15}$N) were determined using a gas source isotope ratio mass spectrometer (ThermoElectron Delta V Advantage) connected to the elemental analyzer by a ThermoElectron Conflow III. The isotope ratios were reported in $\delta$ notation ($\%\epsilon$) relative to Vienna Pee Dee Belemnite standard (V-PDB) for carbon and Air-N for nitrogen according to:
\[ \delta X \ [\%e] = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \]  

(1)

where \( X \) is \(^{13}\)C or \(^{15}\)N, and \( R \) is \(^{13}\)C/\(^{12}\)C or \(^{15}\)N/\(^{14}\)N. Samples were analyzed in duplicate with an average of 0.02\%e analytical differences (mean difference between all duplicates). The IRMS was calibrated using international NIST standards as needed and the calibration checked before and after each run using working standards consisting of freeze-dried, ground spinach leaves and cornhusks.

**Statistical analysis**

Nested analysis of variance (ANOVA; stations nested within streams, and streams within stream networks) with Turkey’s HSD (\( p < 0.05 \)) was performed on sediment organic content and microbial biomass log transformed (n+1) data to determine differences across spatial scales (JMP 10 and MINITAB 16). We reported biomass and abundance per gram of fresh weight sediment, instead of the customary dry weight of sediment, because the sediments varied greatly in their percent water content, violating the assumption necessary for standardizing data to sediment dry weight (that is, sediment dry weight only varies with sample size) (Schallenberg and Kalff 1993).

Relationships among variables were investigated using linear regression and multiple regression analysis (MINITAB 16). We tested data for normality with the Shapiro-Wilk test and homogeneity of variance with Bartlett test and applied appropriate transformations as needed. For multiple linear regression analysis, predictor variables were selected using the ‘best subsets’ algorithm in MINITAB. This algorithm fits a small fraction of all possible regression models and reports the ‘best subset’; we identified the best model based on several selection criteria including adjusted \( r^2 \) and Mallows Cp. We used the structural equations modeling (SEM), more specifically
path analysis, to further explore the influence of environmental variables on microbial biomass. SEM is a multivariate statistical technique that tests the importance of pathways in hypothesized models, and allows comparison of models to experimental data (Mitchell 1992). Standardized regression coefficients between variables were calculated and plotted as path coefficients on path diagrams constructed for microbial biomass. These path coefficients can be used to determine the direct and indirect impacts of environmental variables on the dependent variable. The SEM was performed in SAS Structural Equation Modeling for JMP 10. Natural log transformed (ln + 1) PLFA relative abundance data subjected to principal component analysis (PCA) to identify patterns of variation in the microbial community structure across spatial scales and stream order gradient. PCA was performed for the combined data set of Neversink and WCC networks (SPSS 19). PLFA profiles were interpreted using a functional group approach (Findlay and Dobbs 1993).

RESULTS

Water chemistry and sediment organic content

Water temperatures, at the time of sampling, were similar for all streams (Table 1). While conductivity was only measured for two streams in the White Clay Creek watershed, it is clear that stream water within the Neversink watershed had significantly lower conductivity. Sediment % C and % N showed a complex spatial pattern with sediments from the 1\textsuperscript{st} order streams LSB and WTR showing significantly greater C and N content than all other streams except the WCCW and no significant differences among WCCW and all other streams (Fig. 2a, 2b). Sediment C:N ratios showed several patterns. In general, C:N ratios were higher in 1\textsuperscript{st} and 2\textsuperscript{nd} order streams and lower in 3\textsuperscript{rd} and 5\textsuperscript{th} order stream sediments. In addition, C:N ratios were generally higher in the
White Clay Creek watershed (range: 16.6 – 9.1) compared to the Neversink watershed (range: 12.99 – 5.62) (Fig. 2c).

Total microbial biomass

Total microbial biomass ranged from 6.77 ± 0.75 to 52.41 ± 4.87 nmol PLP g\(^{-1}\) fresh weight sediment (Table 1). ANOVA showed that White Clay Creek sediments contained significantly greater microbial biomass than Neversink River sediments (\(p = 0.002\); Table 2, Fig. 3a) and that there were significant differences among streams within watersheds (Table 2, Fig. 3b). Variability in sediment microbial biomass among stations within streams ranged from moderate (~10%) to 2-fold and showed low to moderate (C.V. = 5.32% to 82.65%) within station variability (Fig. 3c); we did not detect any consistent pattern of higher biomass by station within a reach (upstream vs. midstream vs. downstream stations). Prokaryotes comprised between 58 and 96% of total biomass with casual observations indicating that streams with open canopies (WSB and NRC) showed the highest contribution of eukaryotes to total microbial biomass. Bacterial abundance ranged from \(2.03 \times 10^{8}\) to \(1.68 \times 10^{9}\) cells g\(^{-1}\) fresh weight of sediment. In general, streams from the White Clay Creek watershed showed higher total microbial biomass, percent prokaryotes and bacterial abundance than those within the Neversink watershed (Table 1).

Multiple linear regression analysis indicated that sediment percent carbon content, percent water content, C:N ratio and sediment surface area explained approximately two-thirds of the variation observed in sedimentary microbial biomass (Table 3, Model 7). Path analysis was used to investigate the relationships among these variables and indicated that percent carbon content, percent water content, and C:N ratio had significant direct effects on biomass and that sediment surface area was correlated, to a greater or lesser extent, with carbon content, percent water
content, and C:N ratio (Fig. 4). Two models were investigated, one constrained and a second unconstrained, to investigate the theoretical linkage and directionality among the variables. The constrained model links sediment surface area indirectly to biomass via its direct effects on sediment carbon and water content while the unconstrained model links surface area indirectly to biomass via its correlations with sediment carbon content, water content and C:N ratio; these two models yielded very similar results and we present only the unconstrained model. Percent carbon content showed the greatest direct effect ($r^2 = 0.393$) as well as substantial indirect effects via its correlation with percent water content and C:N ratio (Fig. 4). Combined, the direct and indirect effects of carbon accounted for ~61% of the variation in total sediment microbial biomass. Similarly, percent water content and C:N ratio accounted for 56% and 37%, respectively, of the variation in total sediment microbial biomass. Sediment surface area via indirect effects accounted for ~12% of total sediment microbial biomass.

**Microbial community structure**

The major component of variation in microbial community structure of stream sediments was related to the proportions of prokaryotes and eukaryotes within communities (Fig. 5 & 6). The 5th order Neversink River and the 1st order stream WSB showed the greatest relative abundance of fatty acids indicative of phototrophic and heterotrophic eukaryotic microorganisms (18:3ω3, 20:5ω3, 20:4ω6, 18:2ω6, 16:1ω13t); samples from these streams showed large negative loadings along the PC1. All other streams within both systems showed greater relative abundance of bacterial fatty acids (cy17:0, cy19:0, a17:0, i17:0, i15:0, br17:1a and 10me16:0). The assignment of the relative contribution of bacteria and microeukaryotes to total biomass as the major component of variation based on the high correlation ($r^2 = 0.88$) between PC1 factor scores
and the percentage that microeukaryotes comprise of total microbial biomass (Fig. 6) and is likely related to canopy cover as the NSR and WSB stations were observed to have the most open canopies. Ignoring the two stations with open canopies, the variation in community structure among 1st order streams, for the most part, bounds the variation within the watershed. PC 2 separated streams from Neversink watershed, except the 5th order Neversink River, from all stations within the White Clay Creek watershed. In addition, we observed overlapping PC1 and PC2 scores for sediments from the two Neversink 3rd order streams (Pigeon and Biscuit Brooks) and their 1st order tributaries, indicating similar sediment microbial community structure for these streams. In contrast, microbial community structure of sediment from several of the 1st order streams within the White Clay Creek system showed significant differences among themselves and with White Clay Creek sediments.

*Bacterial community structure*

PCA of bacterial fatty acids profiles separated the two watersheds along the PC1 with all White Clay Creek watershed samples having positive PC1 scores and all Neversink watershed samples having negative PC1 scores, and PC1 scores for all streams within a watershed being similar to at least one other stream within that watershed (Fig. 7). PC 2 separated samples from Neversink River from all other streams in its watershed and separated samples from Watercress Spring and Dirty Dog Spring from all other streams in the White Clay Creek watershed.
DISCUSSION

Stream networks are highly dynamic ecosystems with inherent spatial heterogeneity. This spatial heterogeneity has important implications on the functions, distribution and composition of associated microbial communities. In this study, sediment microbial community structure in the fourteen streams displayed distinct regional scale variations (hundreds of kilometers: i.e., watersheds) and among-stream variations at the scale of hundreds of meters within a watershed, as well as along a stream order gradient. In addition, clear differences in bacterial community structure among streams and between watersheds were documented. These findings extend previous studies examining microbial community structure over regional scales indicating that habitat and geographical distance are important in structuring microbial communities (Hullar et al. 2006; Findlay et al. 2008).

PCA indicated that the benthic microbial and bacterial communities of the 1\textsuperscript{st} and 3\textsuperscript{rd} order streams in the Neversink system were relatively homogenous (Fig. 5 & 7). These streams were fully shaded and received appreciable inputs of terrestrial organic matter that could serve as stable carbon source for bacterial communities that comprised the largest proportion of total microbial communities in headwater streams. The separation of Neversink River, based on microbial community structure, from 1\textsuperscript{st} and 3\textsuperscript{rd} order streams within the Neversink system is related to greater eukaryotic contribution to total microbial biomass in Neversink River. Field observations showed active dense filamentous green algal streamers that were particularly abundant at the NSR station, which has the most reduced canopy cover. These observations were generally consistent with the predominance of chrysophyte and chlorophyte algal biomarkers (18:3\omega3, 20:5\omega3,
16:4\(\omega1\) in describing the variation in microbial community structure among samples from the Neversink system (Fig. 5). This shift in community structure is indicative of increasing exposure to greater irradiance along forested stream channels (i.e., as stream order increases, the amount of stream surface shaded by riparian trees decreases and suggests the potential for decreased importance of allochthonous detrital carbon and increased importance of autochthonous production downstream; Vannote et al. 1980). Thus, headwater streams provide distinctive habitats that shape their characteristic microbial communities in a way that is different in several ways from larger streams. In contrast, the entire benthic microbial community as well as bacterial community sampled in WCC network displayed high among-streams variation among 1\(^{st}\) order streams, while bacterial communities within sediments from the 3\(^{rd}\) order WCC were the most similar to those found in the two 2\(^{nd}\) order streams. The reaches sampled in two 1\(^{st}\) order streams, WTR and WSB, were located in areas where the forest canopy was less dense, which allowed local increases in light availability. The separation of these streams from all other stations in forested WCC system is consistent with the increased contribution of phototrophic microeukaryotes to total microbial biomass and the importance of algal lipid markers (20:4\(\omega6\), 20:5\(\omega3\), 18:2 \(\omega6\), 18:3\(\omega3\)) in describing the variation in microbial community structure (Fig. 5). This suggests that discontinuity in corridors of vegetation along streams and/or modern mosaics of land uses may alter the degree of autotrophy or heterotrophy of a stream. Studies have attributed greater algal biomass in forested watersheds to local increases in light availability (Hill and Harvey 1990; Quinn et al. 1997; Kiffney et al. 2004).

At the regional scale, our data indicated nonrandom spatial variations in total microbial and bacterial communities supporting current evidence for spatial variation in microbial community structure (Martiny et al. 2006). This pattern was the most evident in bacterial PLFA
profiles of community structure, which showed that the major variation in sedimentary bacterial community structure occurred at the watershed level and that the differences among streams with similar general geologic features, light availability and terrestrial vegetation within a watershed were not significant with respect to the major component of variation (Fig. 7). Our data indicated that the composition of microbial communities were sensitive to watershed scale processes. Our findings corroborated those of Findlay et al. (2008) who reported unique streambed communities for each of three biomes. A major difference between our study and that of Findlay et al. (2008) was that the two watersheds examined in this study occurred within the same biome (Eastern Deciduous Forest). This implies that spatial variability in microbial communities occurred at a variety of spatial scales, ranging from the diversity in an individual environmental sample to the diversity assessed across multiple biomes.

But what processes generates these patterns? The Baas-Becking hypothesis for microbial taxa postulates that, ‘everything is everywhere, but, the environment selects’ (Bass-Becking 1934). The claim that ‘the environment selects’ implies that contemporary environmental variation (multiple habitats) maintain distinctive microbial composition. However, the variation in community structure at regional scales may involve multiple causal pathways. For instance, differences in watershed characteristics such as water chemistry, flow regime, temperature, point source inputs, etc., may generate differences in DOM and nutrient qualities and quantities, which in turn cause variation in microbial productivity and community structure (Battin et al. 2008). Also, the proximal causes for the observed variation in streambed microbes between the two watersheds quantified by PCA may be due, in part, to land-cover differences as the Neversink watershed is 95% forested while the WCC watershed is a mixture of pasture land and forest. Also, our data suggested significant differences in conductivity and sediment C:N ratios between the
two watersheds. A review of microbial biogeography studies showed that spatial distributions over small scales often reflect local environmental heterogeneity (reviewed in Martiney et al. 2006). For example, the distribution of bacterial communities in soils (Ramette and Tiedje 2007) and in water column and surface sediments in lakes (Kondo and Butani 2007) at meter to kilometer scales correlated with environmental heterogeneity, whereas the distribution of pseudomonads from undisturbed pristine soils sites (Cho and Tiedje 2000) and hotspring archaeon *Sulfolobus* assemblages (Whitaker et al. 2003) in similar habitats separated by >10,000km correlated with geographical distance. These results indicate that environmental heterogeneity seems to influence microbial community at small spatial scales, whereas at larger spatial scales (>10,000km), geographical distance can overwhelm effects of environmental heterogeneity. Interestingly, studies that sampled at intermediate spatial scale (10 – 3000km) detected the influence of both environmental heterogeneity and geographical distance on microbial biogeography (Green et al. 2004; Yannarell and Triplett 2005). In our study, a hierarchical pattern of overall similarity emerged with the highest similarity found among samples collected within the same stream, especially within the same station, followed by similarities among samples collected from different streams with similar general geologic features and terrestrial vegetation within the same watershed, and finally similarities among samples collected from different watersheds. This implies that the greatest variation in microbial and bacterial community composition in streams occurs at the largest spatial scales.

Total microbial biomass and bacterial abundances for both watersheds were within the range of published microbial biomass for temperate freshwater sediments (Bott and Kaplan 1985; Sutton and Findlay 2003; Findlay et al. 2008) but lower than that reported for an impacted, channelized riverine system in central Ohio (Langworthy et al. 1998). If discontinuities in stream
geomorphology and hydrology occur between streams of different order as predicted by the river continuum concept (Vannote et al. 1980; Benda et al. 2004), one would expect corresponding changes in microbiota and ecosystem processes. However, in our study, microbial biomass did not correlate with increasing stream order as might be expected by the river continuum hypothesis and biomass levels did not differ significantly at all stations within streams for both watersheds. Ferris et al. (2003) reported similarities in bacterial cell densities from three separate riffles in each of three streams investigated. In contrast to reach-scale similarity in microbial biomass, our data showed significant differences at the stream and between watersheds scales (Fig 3).

Significant differences in microbial biomass of streambed sediments among streams have been reported in coastal plain, temperate to tropical evergreen forest headwater streams (Findlay et al. 2002; Gao et al. 2005; Findlay et al. 2008) and high alpine streams (Battin et al. 2004). Overall, the magnitude of within stream variation was small compared to the variability noted among streams and between watersheds and this suggests that microbial biomass within stream reaches is relatively tightly constrained. Path analysis indicated that these environmental constraints were percent sediment carbon content, percent water content, C:N ratio and sediment surface area. These findings are consistent with previous studies (Bott and Kaplan 1985; Findlay et al. 2002; Fierer et al. 2007b). The model revealed that the primary direct controls on microbial biomass in this study were sediment organic carbon, C:N ratios and sediment water content (Fig.4).

Utilization of organic carbon by stream communities can be a measure of ecosystem productivity, while the concentration of organic carbon reflects a combination of several biogeochemical processes (Hedges 1992; Wang et al. 2007). As such, sediment organic carbon may influence microbial biomass through its quantity, quality or a combination thereof. This study revealed that sediment organic carbon influenced total microbial biomass both directly and
indirectly via other correlated variables. Previous studies of stream sediments and terrestrial soils have shown that quantities of carbon can significantly influence microbial biomass (Schallenberg and Kalff 1993, Steenworth et al. 2002; Fierer et al. 2007; Nemergut et al. 2010). In addition, Findlay et al. (2002) showed that variation in quality of sediment detritus, as measured by C:N ratio, was negatively correlated with bacterial abundance, while Schallenberg and Kalff (1993) found variable results (either negative or no correlation) in lake sediments. Our results showed that total microbial biomass (and bacterial abundance, data not shown) was positively correlated with both sediment organic carbon and C:N ratio. The cause of the difference between our findings and those of previous researchers is not known, however, within our system there is a positive correlation between sediment organic carbon and C:N ratio while there was either no relationship between FBOM organic carbon and C:N ratio (Fierer et al. 2007) or the relationship between carbon quantity and quality was not determined (Schallenberg and Kalff 1993; Findlay et al. 2002).

Another important source of variation in this study was sediment percent water content, which has been implicated by other investigators in studies of microbial communities (Doran 1987; Schallenberg and Kalff 1993). Aqueous connectivity within sediment particles allows nutrient and substrate transfer between particles and provides microorganisms with a continuous supply of nutrients and means to move to more favorable locations (Treves et al. 2003). Schallenberg and Kalff (1993) showed that percent water content was the single most important factor explaining sediment bacterial biomass in a series of lakes that differed greatly in sediment grain sizes.

Sediment surface area indirectly affects microbial community biomass via changes in percent organic carbon, C: N ratios and percent water content (Fig.4). Sediment grain could
generate different micro-habitats and increase microscale environmental heterogeneity which has been observed to structure microbial soil biomass and community structure (Treves et al. 2003). In addition, it has been noted that grain size, through its effects on flow rates and availability of nutrients (Bott and Kaplan 1985; Albrechtsen and Winding 1992; Woessner 2000; Vervier et al. 1992) and quantity and quality of organic carbon (Bott and Kaplan 1985; Kaplan and Newbold 2000; Wilcox et al. 2005), can influence microbial biomass.

At the regional scale, sedimentary microbial biomass and community structure from White Clay Creek tended to be different from those in Neversink. There are several factors that could account for these regional effects. White Clay Creek and Neversink watersheds differ in many aspects, including geology (Newbold et al. 1997; Lawrence et al. 2001), landcover and use (Newbold et al. 1997; Lovett et al. 2000; 2002), anthropogenic impacts such as acid deposition (Baldigo and Lawrence 2000), and streamwater chemistry (Newbold et al. 1997; Lawrence et al. 2001 and this study). It is reasonable that these environmental factors could influence stream microbial biomass and community structure through a variety of mechanisms.

In conclusion, the present study indicated that local environmental factors strongly influence sediment microbial biomass and that the magnitude of within stream variation in microbial biomass was small compared to the variability noted among streams and between watersheds. Our results reveal regional-level patterns in microbial community structure and suggest that regional scale environmental factors influence the biogeography of microbes.
Acknowledgments

Sherman Roberts, Michael Gentile and Janna Brown assisted in sample collection and processing. Chirstina Staudhammer provided invaluable advice on the application of path analysis; however, the authors take full responsibility for the application and interpretation of all statistical analyses. Funding for this project was provided by the National Science Foundation DEB-0516235.
LITERATURE CITED


Figure 1. Sampling scheme used to examine microbial biomass and community structure across multiple spatial scales in two watersheds. Sampling within the Neversink watershed consists of four 1st order streams; Biscuit Brook and Pigeon Creek tributaries (Biscuit Brook Tributary A and B [BBTA, BBTB], Pigeon Creek Tributary A and B [PBTA, PBTB], 1a, 1b, 1c, 1d, respectively), two 3rd order streams (Biscuit Brook [BBR] and Pigeon Creek [PBR], 3a, 3b, respectively) and one 5th order stream (Neversink River [NRC]). Sampling within the White Clay Creek watershed consists of four 1st order streams (Ledyards Spring Branch [LSB], Water Cress Spring [WTR], Dirty Dog Spring [DDS] and Walton Spring Branch [WSB], 1e, 1f, 1g, 1h, respectively), two 2nd order streams (East and West Branch White Clay Creek [WCWE, WCCW], 2a, 2b, respectively) and one 3rd order stream (White Clay Creek [WCC]). Sketches of watersheds are not drawn to scale. Each eclipse represents a reach, which contained 3 stations, each of which was sampled 3 times.
Figure 2  Variation in sediment (a) percent carbon, (b) percent nitrogen and (c) C:N ratio by stream, order (1st to 3rd/5th order from left to right) and watershed. Vertical bars denote 0.95 confidence intervals. Streams not connected by a horizontal line are significantly different (p = 0.05, Tukey’s Wholly Significant Difference).
Figure 3. Microbial biomass (mean ± SD) of White Clay Creek and Neversink watershed sediments at three spatial scales: a; watershed, b; stream and c; station. Stream order (or average order for watershed values) are indicated as: black = 1st order, dark gray = 2nd order, light gray = 3rd order, open = 5th order.
Figure 4. Path diagrams describing the structure of the relationship between sediment microbial biomass and % Carbon (%C), % water content, C:N ratios (C:N) and sediment surface area (SSA). Single-headed arrows indicate casual paths; numbers on arrows are path coefficients (standardized regression coefficients) indicating the relative strength of each path leading to a given response variable. Double-headed arrows represent the correlations among the predictor variables. Arrows connecting environmental variables to the independent variable (microbial biomass) indicate direct effects, while environmental variables linked to the independent variable via other environmental variable constitute indirect effects. Path coefficients calculated by SAS Structural Equation Modeling for JMP 10. *= P <0.01, **=P <0.001
Figure 5. Principle Component Analysis of stream sedimentary microbial community structure of White Clay Creek (open circle) and Neversink (open square) watersheds. The percent variation explained by each axis is indicated on the respective component axis. Identified fatty acids had component loadings of $>|0.5|$ with strong influence on the pattern of variation among samples along the respective component axes. Site abbreviations are as described in the legend to Fig.1.
Figure 6. Relationship between Principle Component Analysis factor 1 score and the calculated percentage that microeukaryotes contribute to total microbial biomass for all stream samples.
Figure 7. Spatial variation in sedimentary bacterial community composition in WCC and NRC watersheds by PLFA analysis after removal of fatty acids assigned a priori to the functional group microeukaryotes and those known to be common to both bacteria and microeukaryotes from the PLFA profiles. Symbols- WCC (circle), NSR (square). Site abbreviations are as described in the legend to Fig.1.
Table 1. Microbial biomass, water chemistry and sediment organic content of White Clay Creek and Neversink watersheds.

<table>
<thead>
<tr>
<th>Watershed &amp; stream</th>
<th>Biomass/PLP (nmol g⁻¹ fresh wet wt)ᵃ</th>
<th>Bacterial abundance (g⁻¹ fww)ᵇ</th>
<th>% Eukaryotic/prokaryoticᶜ</th>
<th>Cond (µS/cm)</th>
<th>Temp (°C)</th>
<th>δ¹³C</th>
<th>δ¹⁵N</th>
<th>% C</th>
<th>% N</th>
<th>C:N ratioᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>White Clay Creek</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDS</td>
<td>14.80 ±2.81</td>
<td>5.67 x 10⁸</td>
<td>4/96</td>
<td>176.8</td>
<td>ND</td>
<td>-26.89 ±0.65</td>
<td>2.23 ±0.20</td>
<td>0.33 ±0.01</td>
<td>0.02 ±0.55</td>
<td>14.14 ±3.18</td>
</tr>
<tr>
<td>WSB</td>
<td>30.34 ±9.81</td>
<td>8.24 x 10⁸</td>
<td>31/69</td>
<td>ND</td>
<td>ND</td>
<td>-29.36 ±0.79</td>
<td>3.04 ±0.25</td>
<td>0.58 ±0.02</td>
<td>0.04 ±0.54</td>
<td>12.98 ±2.53</td>
</tr>
<tr>
<td>LSB</td>
<td>43.29 ±19.60</td>
<td>1.56 x 10⁹</td>
<td>9/91</td>
<td>ND</td>
<td>ND</td>
<td>-27.65 ±0.46</td>
<td>2.49 ±3.78</td>
<td>2.96 ±0.37</td>
<td>0.31 ±0.51</td>
<td>12.77 ±5.12</td>
</tr>
<tr>
<td>WTR</td>
<td>52.41 ±4.87</td>
<td>1.68 x 10⁹</td>
<td>20/80</td>
<td>ND</td>
<td>ND</td>
<td>-28.25 ±0.59</td>
<td>2.27 ±2.02</td>
<td>3.36 ±0.15</td>
<td>0.21 ±0.34</td>
<td>16.45 ±2.09</td>
</tr>
<tr>
<td>WCWE</td>
<td>22.13 ±3.38</td>
<td>8.26 x 10⁸</td>
<td>6/94</td>
<td>ND</td>
<td>ND</td>
<td>-27.23 ±1.18</td>
<td>2.67 ±0.83</td>
<td>1.15 ±0.04</td>
<td>0.07 ±0.37</td>
<td>16.27 ±2.75</td>
</tr>
<tr>
<td>WCCW</td>
<td>26.89 ±2.52</td>
<td>1.01 x 10⁹</td>
<td>6/94</td>
<td>ND</td>
<td>16.2</td>
<td>-26.81 ±0.61</td>
<td>3.27 ±1.52</td>
<td>1.73 ±0.11</td>
<td>0.11 ±0.37</td>
<td>16.59 ±2.71</td>
</tr>
<tr>
<td>WCC</td>
<td>12.12 ±4.95</td>
<td>4.25 x 10⁸</td>
<td>11/89</td>
<td>205</td>
<td>16.1</td>
<td>-26.09 ±1.26</td>
<td>-4.70 ±0.55</td>
<td>0.57 ±0.04</td>
<td>0.06 ±5.03</td>
<td>9.08 ±2.96</td>
</tr>
<tr>
<td><strong>Neversink</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBTA</td>
<td>6.99 ±2.39</td>
<td>2.27 x 10⁸</td>
<td>19/81</td>
<td>35.1</td>
<td>15.4</td>
<td>-27.32 ±1.01</td>
<td>1.71 ±0.16</td>
<td>0.36 ±0.01</td>
<td>0.03 ±1.08</td>
<td>11.15 ±2.30</td>
</tr>
<tr>
<td>BBTB</td>
<td>10.95 ±3.74</td>
<td>3.34 x 10⁸</td>
<td>23/77</td>
<td>32.4</td>
<td>14.8</td>
<td>-25.76 ±1.19</td>
<td>2.29 ±0.21</td>
<td>0.34 ±0.01</td>
<td>0.03 ±0.55</td>
<td>10.25 ±3.15</td>
</tr>
<tr>
<td>PBTA</td>
<td>12.18 ±3.78</td>
<td>3.92 x 10⁸</td>
<td>18/82</td>
<td>25.6</td>
<td>16.2</td>
<td>-27.09 ±0.67</td>
<td>2.61 ±0.68</td>
<td>0.72 ±0.04</td>
<td>0.05 ±0.35</td>
<td>12.99 ±3.10</td>
</tr>
<tr>
<td>PBTB</td>
<td>16.42 ±6.96</td>
<td>5.73 x 10⁸</td>
<td>12/88</td>
<td>18.8</td>
<td>15.5</td>
<td>-23.54 ±3.22</td>
<td>3.69 ±1.85</td>
<td>1.18 ±0.11</td>
<td>0.08 ±0.38</td>
<td>11.06 ±3.30</td>
</tr>
<tr>
<td>BBR</td>
<td>8.54 ±1.79</td>
<td>2.87 x 10⁸</td>
<td>16/84</td>
<td>20.8</td>
<td>16.4</td>
<td>-25.17 ±1.70</td>
<td>1.82 ±0.10</td>
<td>0.18 ±0.00</td>
<td>0.03 ±0.30</td>
<td>5.62 ±2.38</td>
</tr>
<tr>
<td>PBR</td>
<td>6.77 ±0.75</td>
<td>2.03 x 10⁸</td>
<td>25/75</td>
<td>24.5</td>
<td>16</td>
<td>-22.97 ±2.83</td>
<td>-0.34 ±0.14</td>
<td>0.16 ±0.01</td>
<td>0.02 ±0.88</td>
<td>6.18 ±3.76</td>
</tr>
<tr>
<td>NRC</td>
<td>13.58 ±4.41</td>
<td>2.95 x 10⁸</td>
<td>42/58</td>
<td>33.5</td>
<td>15.3</td>
<td>-25.41 ±2.05</td>
<td>0.60 ±0.08</td>
<td>0.20 ±0.01</td>
<td>0.03 ±0.71</td>
<td>7.69 ±1.05</td>
</tr>
</tbody>
</table>

ᵃ Mean ± standard deviation (n = 9).
ᵇ Calculated from PLP x % prokaryotic (expressed as decimal fraction) and a conversion factor of 100 nmol PLP = 4 x 10⁹ cells
ᶜ Percentage that microeukaryotic contributes of total microbial biomass, calculated from PLFA profiles (n = 9).
ᵈ Percent that C and N contribute to total sediment elementary atoms (n = 9)
ND= measurement were not taken
Table 2. Nested ANOVA to test the effects of watershed, streams within watershed, and stations within streams on microbial biomass.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watershed</td>
<td>1</td>
<td>5.119</td>
<td>5.119</td>
<td>15.18</td>
<td>0.002</td>
</tr>
<tr>
<td>Stream (Watershed)</td>
<td>12</td>
<td>4.047</td>
<td>0.337</td>
<td>7.38</td>
<td>0.000</td>
</tr>
<tr>
<td>Stations (Watershed*Stream)</td>
<td>28</td>
<td>1.280</td>
<td>0.046</td>
<td>1.46</td>
<td>0.095</td>
</tr>
<tr>
<td>Error</td>
<td>84</td>
<td>2.628</td>
<td>0.031</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Multiple regression analysis (best subsets) for natural log biomass as a function of various physical and chemical stream parameters

<table>
<thead>
<tr>
<th>Model</th>
<th>Vars</th>
<th>R-Sq</th>
<th>R-Sq(adj)</th>
<th>Mallows Cp</th>
<th>SE</th>
<th>%Water</th>
<th>SSA</th>
<th>%C</th>
<th>%N</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>60.7</td>
<td>60.4</td>
<td>30.2</td>
<td>0.2052</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>56.4</td>
<td>56.0</td>
<td>46.3</td>
<td>0.2161</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>65.0</td>
<td>64.4</td>
<td>15.9</td>
<td>0.1943</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>64.6</td>
<td>64.0</td>
<td>17.6</td>
<td>0.1955</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>67.6</td>
<td>66.7</td>
<td>8.4</td>
<td>0.1879</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>66.9</td>
<td>66.0</td>
<td>11.1</td>
<td>0.1899</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>7</strong></td>
<td><strong>4</strong></td>
<td><strong>69.2</strong></td>
<td><strong>68.1</strong></td>
<td><strong>4.3</strong></td>
<td><strong>0.1839</strong></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>68.1</td>
<td>67.0</td>
<td>8.5</td>
<td>0.1872</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>69.3</td>
<td>68.0</td>
<td>6.0</td>
<td>0.1845</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
CHAPTER 3
SEASONALITY IN A STREAMBED MICROBIAL COMMUNITY: VARIATION IN THE
ISOTOPIC COMPOSITION OF LIPID BIOMARKERS

ABSTRACT

Microbial biomass and community structure can show dramatic seasonal variability in temperate
stream ecosystems and understanding links between this variability and in-stream biotic and
abiotic processes is an important goal for stream ecologists. Stable isotopic composition of
organisms and related biomolecules has increasingly become an important tool for ecologists
probing stream processes. To test if the seasonal changes in the structure of stream sediment
microbial communities altered the stable carbon isotope signatures of microbial phospholipid
fatty acids, we collected sediments from White Clay Creek from December 2009 through October
2010. Sedimentary microbial biomass, measured as total phospholipid phosphate, ranged from 10
to 29 nmol PLP g\textsuperscript{-1} dry weight sediment and was significantly correlated with high and low flood
pulse counts, variability in daily flow and dissolved organic carbon (DOC) concentrations.
Principal component analysis of phospholipid fatty acid (PLFA) profiles indicated that the
sedimentary microbial communities displayed seasonal patterns of change as a result of a shift
from dominance of prokaryotes during times of cold water to increased importance of
phototrophic microeukaryotes during times of warm water. This shift was significantly correlated
with seasonal changes in median daily flow, DOC, high and low flood pulse counts and water
temperature. Microbial carbon isotope signatures using compound-specific $^{13}$C analysis of PLFA
showed that both bacterial and microeukaryotic stable carbon isotope ratios were heaviest in the spring and lightest in autumn or winter. Bacterial PLFAs were isotopically depleted on average by 2-5‰ relative to $\delta^{13}$C of total organic carbon suggesting bacteria consumption of terrestrial organic matter. Most bacterial PLFAs were enriched on average by 9-12‰ compared with algal PLFA indicating an uncoupled algal-bacteria system. However, $\delta^{13}$C values of bacterial PLFA of summed feature 1 (16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t) were enriched compared with algal PLFA by 5.7‰ suggesting utilization of autochthonous DOC, along with allochthonous detritus as carbon sources. Our study revealed seasonal fluctuations in microbial biomass, community structure and their lipid isotopic signatures examined in stream sediments, and also demonstrated the potential influences of various hydrological indices on microbial biomass and community composition in lotic ecosystems.
Stable isotope analysis, in particular those of carbon and nitrogen, is a rapidly expanding tool used by ecologists to examine diet (Ben-David et al. 1997; Karlsson et al. 2003), foraging ecology (Rubenstein and Hobson 2004; Cherel et al. 2007), ecophysiological processes (Gannes et al. 1998; Cernusak and Hutley 2011), and trophic position and food-web analysis (Kwak and Zedler 1997; McNabb et al. 2001; Post 2002), as well as evaluating the structure and dynamic of ecological communities (Vander Zanden et al. 1999; Post et al. 2000). Also, stable isotope analysis has been successfully used to assess spatial and seasonal variability in the isotopic composition of organisms in various ecosystems (Riera and Richard 1997; Vizzini et al. 2002; Finlay 2004). Moreover, advances in bulk stable isotope analysis have improved methodological approaches (Tobias et al. 2008) and mathematical models (Logan et al. 2008; Bond and Diamond 2011), enhanced linking processes at multiple scales (Martínez del Rio et al. 2009), and addressed more complex ecological questions, such as determining the primary controls of high variability observed in lotic algal δ¹³C values (Finlay 2004; Ishikawa et al. 2012).

Although, bulk stable isotope analysis has been extensively used in ecology, compound-specific stable isotope analysis is a more robust tool in microbial ecology that enables the molecular specificity and isotopic signature of individual compounds to be exploited concurrently (Boschker et al. 1998). Recent technological advances in the development of gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) for compound specific stable isotope analysis have increased our ability to link microorganisms in environmental samples to utilization of particular growth substrates (Rieley et al. 1991; Dumont
and Murrell 2005). Presently, phospholipid fatty acid (PLFA), amino acids and nucleic acids have been used as biomarker molecules and for compound-specific stable isotope analysis (Boschker et al. 1998; Radajewski et al. 2000). Investigation of carbon isotope signature of individual PLFAs allows for identification of carbon source use by microorganisms. This methodological approach has several appealing advantages including short incubation times and use of trace-level additions (< 5% of total carbon). These advantages arise from the high precision of GC/C/IRMS, which allows for the detection of changes of as little as 4‰ to be considered a significant change in stable isotope ratio. However, this sensitivity requires an understanding of the variation in isotopic signatures of specific biomarker compounds of microbial communities, both temporally and among the various components of the community. As the availability of this advanced technique is relatively recent, studies investigating variations in the compound specific isotopic composition of lipid biomarkers of organic matter and microbial composition are scarce and, to the best of our knowledge, only conducted in estuarine sediments (Zimmerman and Canuel 2001; Dai and Sun 2007); such studies are still lacking in stream sedimentary habitats.

Seasonal dynamics in sedimentary microbial biomass and community structure in lotic ecosystems have been documented (Kaplan and Bott 1989; Smoot and Findlay 2001; Battin et al. 2001; Sutton and Findlay 2003; Hullar et al. 2006). In addition, several environmental variables have been considered to influence microbial community composition in several habitats (reviewed in Horner-Devine et al. 2003). For example, Smoot and Findlay (2001) reported that benthic microbial communities were dynamically responsive to physico-chemical and biological parameters that varied seasonally in a riverine-reservoir ecosystem. In addition, temperature, pH and dissolved organic carbon (DOC) have been associated with temporal shifts in bacterioplankton communities in lakes and streams (Crump et al. 2003; Lindström et al. 2005;
Environmental variables such as climate, topography, underlying geology, inorganic water chemistry and riparian vegetation are more constrained and significantly influence stream processes. Other variables, such as streamflow velocity, light, temperature, quantity and quality of allochthonous and autochthonous inputs (e.g. DOC) and high frequency of storms, can vary dramatically throughout the year and play an important role in structuring the stream communities and hydrological conditions of temperate streams (Kaplan and Bott 1989; Autio 1998; Gremm and Kaplan 1998; Olapade and Leff 2005; Hullar et al. 2006).

Although it is known that seasonal variation in sedimentary microbial community structure occurs in streams (Hullar et al. 2006), there have been no reports describing seasonal variation in carbon isotope signatures of individual PLFAs in relation to temporal changes observed in microbial community structure in stream sediments. In this study, we aimed to investigate if this variation in sedimentary microbial community structure contributes to any observable variation in isotope profiles of microbial PLFAs. Our study approach was to investigate the seasonal differences in microbial biomass, community structure and microbial PLFA isotopic signatures in order to compare patterns in stable isotope profiles and community structure and to assess the relationships to varying hydrological indices. We sampled replicate microbial community within a single reach over an annual cycle in order to constrain potentially confounding environmental variables such as climate, topography, underlying geology, inorganic water chemistry, and riparian vegetation. Seasonal variations in benthic microbial community structure and stable carbon isotope signatures of individual microbial fatty acids were determined using PLFA and compound specific stable isotope analyses, respectively. Annual stream discharge data for 5-year period were obtained from the US Geological Survey Water Resources
database. Our data were subjected to multivariate statistical analyses to characterize flow regime and elucidate the dominant sources of variation and correlation of microbial community structure with seasonal changes in hydro-ecological parameters.

MATERIALS AND METHODS

Study site

The study site was located within the 3rd order reach of 7.3 km² White Clay Creek (WCC) watershed directly adjacent to the Stroud Water Research Center, Avondale, PA. The WCC stream is 2,400m long, occurs within riparian woodlands in the Piedmont Province of southeastern Pennsylvania and northern Delaware and joins the Christina River near the Christina's discharge to the Delaware Bay. Upstream of our sampling station, WCC has a protected riparian zone and drains a 725 ha watershed comprised of approximately 52% of agricultural, 22% of tilled/hayed and 23% of wooded lands (Wiegner et al. 2005; Newbold et al. 1997). Streamflow and streamwater chemistry have been monitored at regular intervals since the 1970s with mean annual stream flow, stream water temperature, and local precipitation of 115 L/s, 10.6°C, and 105 cm y⁻¹, respectively. Streambed sediments consist of clay-, silt-, and sand-sized particles in pools and runs, with gneiss- and schist-derived gravel and cobble in riffles. The dominant tree species reported are beech (Fagus grandifolia), red oak (Quercus rubra), black oak (Quercus velutina) and tulip poplar (Liriodendron tulipifera). Detailed description is given in Newbold et al. 1997.
Study design

At the study site, streambed sediments were collected at monthly intervals (with the exception of January, 2010) over eleven months during December 2009 and October 2010. The stream annual hydrograph showed higher flows during the spring of the year, lowest flows during late summer-early fall and was punctuated by storm-driven high flow events. There were no zero-flow days (i.e., surface water not present or present in isolated pools). Sampling was done pre- and post- high flow events, and during average flow and low flow periods (Fig.1). Sampling periods coincided with summer (June to August 2010), autumn (Sept and Oct 2010), winter (Dec 09, Feb and Mar 2010) and spring (April and May 2010). Samples were delimited with a 100mm diameter Plexiglas ring that was inserted 2 cm deep into the streambed. Plexiglas plates were slipped under and over the ring to effectively trap the sediments and allowed them to be lifted from the streams with minimum disturbance. Sediments in the top 2mm within the ring were transferred with a clean spatula to pre-labeled Whirl-Pak sampling bags and transported on ice to the laboratory. In December 2009, three replicate samples were collected from left-mid-right locations across the stream, however, from February to October 2010 nine samples, three from each station within the reach were collected with stations separated by approximately 10m. In the laboratory, sediments were transferred to a clean plastic weigh boat and thoroughly homogenized, and subsampled for phospholipid and elemental analyses.

Streamflow data and hydrological indices

Daily streamflow data for WCC at Avondale, PA (Lat 39°49′42″, long 75°46′52″) were acquired from the US Geological Survey Water Resources database (http://waterdata.usgs.gov). There is little or no flow regulation and the drainage area is 11.3 m². The flow data consisted of a
5-year period from 1 October 2007 to 30 September 2012. We examined 108 hydrologic indices to identify a subset of biologically relevant indices that can best explain critical attributes of the flow regime and variation in microbial biomass and community structure. These indices were distributed into four categories following Richter et al. (1996) and Poff et al. (1997). These are magnitude (n=79), frequency (n=10), duration (n=12) and rate of change (n=7). Timing of flow such as predictability and constancy, which are known to be sensitive to the length of record used in their calculation (Gan et al. 1991; Clausen and Biggs 2000), were excluded from analysis because of the short period of record used in this study.

Our general approach to hydrological assessment was first to identify a series of ecologically relevant hydrological indices that characterized dominant inter-annual variation in flow conditions and then analyze these ‘unique’ hydrological attributes as the predictors for variation observed in microbial biomass and community structure. The approach involved five steps:

1- Compute values for hydrological indices. We calculated values for each 108 streamflow variables for the WCC for each year of the five-year period. The hydrological indices were distributed into four categories: the magnitude of flow, frequency of occurrence of flows above a given magnitude, duration of flow for specific flow conditions and the rate of change or flashiness of flows (see http://nj.usgs.gov/projects/2454C2R/EcoFlow/definitions.html, Olden and Poff 2003 and Clausen and Biggs 2000 for hydrologic index definitions, and supplementary data for hydrologic index values calculated for this study).

2- Compute inter-annual statistics. We performed data ordination by using principal component analysis (PCA) to elucidate major patterns of intercorrelation among the hydrologic variables and identify relevant subsets of indices that structure the interannual variation. Standardized PCA
based on correlation matrix was obtained by centering and standardization by ‘species’ (in CANOCO 4.5) since hydrological variables were measured in different units. We selected six representative descriptors of the hydrograph as unique ecological relevant hydrological indices that best characterized inter-annual variation in flow conditions; referred to as indicators of hydrological variation (IHV).

3- Compute IHV values prior to sampling. We calculated values for each IHV for 2-week period prior to stream sediment sampling for the sampling year (2009/2010) in order to evaluate the sensitivity of microbial biomass and community structure to certain types of hydrological impacts.

4- Calculate multicollinearity of the IHV. To test whether collinearity existed within the IHV we compared the correlation matrices of the IHV calculated for 2-week period prior to stream sediment sampling and eliminate two highly correlated indices.

5- Compute multiple regression analysis of IHV and environmental variables. Multiple regression analysis (‘best subsets’ in MINITAB 16) was performed on uncorrelated indices of IHV and other environmental parameters measured in this study.

The 108 hydrologic indices for 5-year period used in this study are presented in Appendix 1 of the supplementary data. The results from the biplot of PCA are presented in Appendix 2 of the supplementary data.

Phospholipid fatty acids analysis

Microbial biomass and community structure were determined using phospholipid analysis following the methods of Findlay (2004). Briefly, lipids were extracted from frozen sediment samples in the dark at 4°C in 50ml screw-cap glass tubes with 27ml of a 1:2:0.6 (v/v/v)
dichloromethane-methanol-50 mM phosphate buffer (pH 7.4) solution. The solution was partitioned into organic and aqueous phases with 7.5 ml dichloromethane and 7.5 ml deionized water, after which the organic phase (containing total lipid) was collected through a predried 2V filter (Whatman, Schleicher & Schuell) into 15 ml test tubes and the solvent dried under Nitrogen at 37°C. The dried lipid was dissolved in 2 ml chloroform and two 100 µl subsamples were oxidized with potassium persulfate at 100 °C overnight in sealed ampoules to release orthophosphate. Phosphate content was determined spectrophotometrically (610 nm) using a dye-coupled reaction between ammonium molybdate and malachite green. The remainder of the dissolved lipid was fractionated into neutral, glyco-, and phospholipid with silica gel solid phase extraction chromatography. Phospholipid fatty acids (PLFAs) were converted into their respectively methyl esters by base methanolysis and purified by octadecyl bonded silica gel (C18) reverse-phase column chromatography. Fatty acid methyl esters (FAMEs) were identified and quantified using Agilent gas chromatograph-flame ionization detection (GC-FID). FAME identification was based on relative retention times, coelution with standards, and mass spectral analysis. Individual fatty acids were analyzed for both absolute and relative abundance. Absolute abundance data (µg FAME g\(^{-1}\) dry weight) allowed the determination of functional group biomass within the microbial community, while relative abundance or weight percent data (gram individual fatty acids x gram\(^{-1}\) total fatty acids x 100) allowed the determination of community structure (Findlay and Dobbs 1993). The FAME nomenclature used followed Findlay and Dobbs (1993). Standard nomenclature was used to refer to the fatty acids: the total number of carbon atoms is followed by a colon, and the number of double bonds. The position of the first double bond is indicated by \(\omega\) and the number of carbon atoms from the aliphatic end. For example, the fatty acid 18:2\(\omega\)6, is 18 carbons long, and has two double bonds that occur at the sixth carbon
from the *omega* end of the molecule. The suffixes *c* and *t* specify the *cis* and *trans* configurations of the double bond, respectively. Methyl branching at the *iso* and *anteiso* positions and at the 10th carbon atom from the carboxyl end is designated by the prefixes *i*, *a*, and *10Me*, respectively. The prefix *cy* denotes cyclopropane fatty acids.

*Isotopic Analysis of Biomarker Compounds*

Isotopic analysis of individual FAMEs were determined using a Delta V Advantage (ThermoElectron) isotope ratio mass spectrometer coupled with an Agilent 6890N gas chromatograph via a GC/C III (ThermoElectron) combustion interface. Gas chromatographic separation of FAMEs utilized a BPX70 column (50m x 0.32mm, 0.25µm film thickness, insert source). The separation and combustion of fatty acids are described in detail in Abraham and Hesse (2003). All samples were run in duplicate. Precision for isotopic measurements of individual compounds was ± 0.6‰. Stable isotope composition was expressed in the δ notation with V-PDB as standard:

$$\delta^{13}C = \left[\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right] \times 1000 \quad (1)$$

where $R$ is $^{13}C/^{12}C$ in the samples and standard.

Derivatization of the fatty acids introduced an additional carbon to the molecule altering isotope ratios. Therefore, calculation of the carbon isotope ratio of FAMEs included a correction for the isotope ratio of the methyl moiety to obtain the original isotope ratio of fatty acids using the equations taken from Abraham et al. (1998):

$$\delta^{13}C_{FA} = [(C_n + 1) \times \delta^{13}C_{\text{FAME}} - \delta^{13}C_{\text{MeOH}}]/C_n \quad (2)$$
where $\delta^{13}C_{FA}$ is the $\delta^{13}C$ of the fatty acid, $C_n$ is the number of carbons in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}C$ of the FAME, and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol used for the methylation reaction, which was determined to be -43.23‰.

**Elemental Analysis**

The frozen subsamples for elemental analysis were freeze-dried, finely grounded to provide a homogenous sample, weighed (about 1.5 - 2g) into glass vials, placed uncovered in a desiccator containing 2N HCl and evacuated with a sink aspirator. Fumed samples were freeze-dried, weighed and re-fumed until constant weight. Approximately 35mg of dried sediment sample were weighed in tin boats and analyzed on a Costech 4010 elemental analyzer for $\delta^{13}C$, $\delta^{15}N$, percent carbon and nitrogen, and atomic carbon to nitrogen ratio (C:N). Samples were analyzed at least in duplicate with an average of 0.02‰ analytical differences.

**Statistical analysis**

One-way analysis of variance (ANOVA) with Turkey’s HSD (p < 0.05) was performed on sediment organic content and microbial biomass log transformed (n+1) data to determine temporal differences (QI Macros 2012 for Excel). Significant differences were assessed at an $\alpha$ error level of p = 0.05. To investigate the interrelationships between the biological (microbial biomass and community structure) and hydro-ecological variables, linear regression and multiple linear regression analyses (MINITAB 16) were used. For multiple linear regression analysis, predictor variables were selected using the ‘best subsets’ algorithm in MINITAB. This algorithm fits a small fraction of all possible regression models and selects the ‘best subset’ based on several selection criteria such as Mallow Cp and adjusted $r^2$. 

63
Natural log transformed (ln+1) PLFA relative abundance data were subjected to principal component analysis (PCA; SPSS 21) to identify patterns of seasonal variation in the microbial community structure. PLFA profiles were interpreted with a functional group approach (Findlay and Dobbs, 1993). PCA biplots (CANOCO 4.5) were used to examine major patterns of intercorrelation among the hydrologic variables and identify representative descriptors that structure the interannual variation. Path analysis was used to investigate the relationships among environmental variables and explore the directions of influence of these variables on microbial biomass. The path analysis was performed in SAS Structural Equation Modeling for JMP 10.

RESULTS

Physical and chemistry stream characteristics

Mean monthly flows ranged from 0.61 – 1.22 m$^3$s$^{-1}$ during winter and decreased gradually in spring to an annual low in late summer (0.15 m$^3$s$^{-1}$), before dramatically increasing in October 2010 to 0.55 m$^3$s$^{-1}$ (Fig. 1). The hydrograph was also characterized by spikes in flow, associated with storm events, where mean daily flow can increase to as high as 52 m$^3$s$^{-1}$. During the study period, water temperature varied from a minimum of 2.8°C in December to a maximum of 20.3°C in August showing clear annual trends typical of temperate streams (Table 1). DOC concentrations varied throughout the year, with the annual low in mean monthly concentration occurring in late winter (February 2010) and highest monthly mean concentration observed in October 2010. There was no significant correlation between mean monthly streamflow and mean monthly DOC concentration.
There was a high degree of inter-correlation (either positive or negative) among many of the 108 calculated hydrologic indices for White Clay Creek over the 5-year period examined. Principal component analysis was used to reduce the number of variables and to determine which parameters best explained the differences among each year. Representative descriptors of the hydrograph that were significantly associated with inter-annual variations were used as putative drivers of microbial biomass; these were high flood pulse count (FHC1), mean daily flows (MDF), low flood pulse count (FLC), median daily flow (MQ50), base flow index (MBI) and variability in daily flow (MVD1).

**Total Microbial Biomass**

Total microbial biomass, measured as phospholipid phosphate, ranged from $10.05 \pm 6.95$ to $28.96 \pm 12.13$ nmol PLP g$^{-1}$ dry weight sediment and increased during spring to its peak in June before declining during summer and early fall (Table 1). Total sediment microbial biomass was significantly greater during May and June compared to December and February. The proportion that microeukaryotes comprised of total sediment microbial biomass was significantly smaller (6 – 7%) during the winter (December 2009 and February 2010) compared to remaining months (21 – 30%) (Table 1). Bacterial abundance estimates ranged from $3.78 \times 10^8$ in December 2009 to $9.19 \times 10^8$ cells g$^{-1}$ sediment (dry wt) in June 2010 and showed a similar seasonal pattern as observed for total microbial biomass.

**Microbial Community Structure**

Principal component analysis of PLFA profiles revealed that the samples formed three clusters that suggest a seasonal pattern in microbial community structure in WCC sediments (Fig.
2). Samples from December and February formed one cluster and were taken during a period of low water temperatures (mean daily temperatures; 2.8 - 3.8 °C) and high stream flows (mean daily flows; 0.61 – 1.14 m³ s⁻¹) (Table 1). Samples from March and April formed a second cluster and were taken during a period of moderate water temperatures (7.6. - 12.3 °C) and high stream flows (0.72 – 1.22 m³ s⁻¹). Samples from May through October formed the third cluster and were taken during a period of moderate to high water temperatures (11.8. - 20.3 °C) and low to moderate stream flows (0.15 – 0.55 m³ s⁻¹). Samples from December and February showed positive PC1 component scores and were enriched in PLFAs a15, cy17:0, 10me16, cy19:0, i15:0, br17:1a and i17:0. These fatty acids are typically considered bacterial in origin, and can be indicative of several bacterial functional groups. Samples collected in March and April had negative PC1 component scores and were enriched in PLFAs 16:1ω13t, 20:5ω3 and 16:4ω1. These fatty acids are consistent with the presence of phototrophic microeukaryotes. Samples collected from May through October had positive PC2 component scores (December through April samples showed negative PC2 scores) and were enriched with PLFAs 20:4ω6, br19:1a, 18:2ω6, 18:3ω3, 15:0 and 18:1ω9. The even-chain length fatty acids in this group are consistent with the presence of heterotrophic microeukaryotes. The importance of the seasonal variation in proportions of prokaryotes and eukaryotes to the patterns of change within microbial community structure was further accentuated by the high correlation (r² = 0.54) between PC1 factor scores and the percentage that microeukaryotes comprise of total microbial biomass (Fig. 3).

**Relationships between microbial biomass, community structure and environmental variables**

Analysis of Pearson correlation coefficients between indicators of hydrological variation, temperature and DOC concentrations showed that base flow index (MBI) and mean daily flows
(MDF) were highly correlated \((r > 0.7)\) with MQ50, MVD1 and FLC (Table 2) and MBI and MDF were removed prior to multiple linear regression analysis of the relationship between total microbial biomass and environmental descriptors. Multiple linear regression analysis indicated that high flood pulse count provided the best single variable model explaining the variation in total sediment microbial biomass (Table 3, Model 1; adjusted \(R^2 = 0.33\)). Best subset multiple linear regression indicated that a combination of high flood pulse count, low flood pulse count and variability in daily flow best explained the observed annual variation in sedimentary microbial biomass in WCC sediments (Table 3, Model 5; adjusted \(R^2 = 0.49\)).

A simple linear regression analysis indicated that the percentage that microeukaryotes comprise of total microbial community explained approximately half of the variation observed in PC1 scores (Fig. 3). The multiple component model to explained most of the variation observed in sedimentary microbial community structure indicated that combination of temperature, DOC, MQ50, FLC and FHC1 explained 93% of the variation (Table 4, Model 9). Path analysis showed that temperature, median daily flow and high flood pulse count were negatively correlated with PC1 scores while DOC concentration and low flood pulse count showed positive correlations with this descriptor of seasonal variations in community structure.

General patterns and seasonal dynamics in PLFAs stable carbon isotopes

Phospholipid fatty acid isotope ratios ranged from a low of -43.66 ± 1.41 (20:5w3, September) to a high of -28.23 ± 1.45 (i16:0/15:1, February) (Table 5). Total sediment organic carbon (TOC) stable isotopic ratios averaged -26.50‰ ± 0.73‰ (although this data set is incomplete as 4 months worth of samples were lost in transit; Table 1). Differences in isotopic signatures between bacterial PLFA and TOC ranged between 2 - 5‰, depending on the fatty acid.
The phototrophic microeukaryotic marker 20:5w3 showed $\delta^{13}$C values of -36 to -44‰ and differences in isotopic signatures between 20:5w3 and TOC were substantial and ranged from 9 - 17‰ (Fig. 4).

Principal component analysis of compound-specific stable carbon isotope ratios profiles of the White Clay Creek sedimentary microbial community indicated that for much of the year (December, March, May and June) profiles were similar (Fig. 5). However, three months (April, July and September) showed significant variation from the majority of the samples and two months (February and October) showed high variability among replicates. The samples collected in April showed positive PC1 scores while sediment samples collected in summer (July and September) showed negative PC1 scores. PC1 scores for samples collected in October spanned those for July and September and December, March, May and June. Similarly, February samples showed PC1 scores that spanned those from April and December, March, May and June.

**DISCUSSION**

Microbial biomass, community structure and PLFAs stable carbon isotope ratios were seasonally dynamic in White Clay Creek sediments. Total microbial biomass was lowest during winter months and peaked during May and June, a period of moderate temperatures and stream flow. Microbial community structure was dominated by prokaryotes during winter months but then showed an increase in the relative abundance of several PLFA (16:1w13t, 20:5w3, 16:4w1) typically associated with phototrophic microeukaryotes during March and April. The relative importance of microeukaryotes remained high during May through October, however, PCA of
PLFA profiles suggested that heterotrophic microeukaryotes gained in relative importance during this period. Compound specific stable isotope analysis revealed that most bacterial PLFAs (e.g. i15:0, a15:0) closely tracked $\delta^{13}C$ values of sediment total organic carbon, and varied little over the course of the study. The PLFA 20:5$\omega$3 was the most depleted of all fatty acids and was one of most variable ranging from a low of -43.89 in December to a high of -36.17 in April.

Total microbial biomass and bacterial abundance for WCC exhibited seasonal fluctuations and closely reflected variations in stream physicochemistry, indicating a tightly coupled response by microbial biomass to environmental variables. We found that a significant amount of the variance (49%) in total microbial biomass was explained by a combination of high and low flood pulse counts, variability in daily flow and DOC concentration. This suggests that flood disturbance frequency (as depicted by high and low flood pulse counts) and flow rate (mean or median flow) should be major factors influencing benthic microbial productivity in streams. Our results extend to total microbial biomass the findings of Poff et al. (1990), who demonstrated that benthic algal biomass was significantly impacted by current regime. For example, sediment microbial biomass in WCC showed peak total biomass in late spring/early summer when mean monthly flows were moderate, variability in daily flow was low and DOC was in steady supply. These findings extend to heterotrophic microbial communities earlier reports that current regime can structure the development of benthic communities in streams (Poff and Ward 1989; Biggs 1996; Clausen and Biggs 1997).

Total microbial biomass and bacterial abundance were within the range of published microbial biomass for temperate freshwater sediments (Bott and Kaplan 1985; Sutton and Findlay 2003; Findlay et al. 2008) but lower than that reported for an impacted, channelized riverine system in central Ohio (Langworthy et al. 1998; 2002) and contaminated subsurface riverine
sediments (Mosher et al. 2006). Total microbial biomass was low and microeukaryotic contribution to total biomass was significantly reduced during the winter when water temperature was below 4°C. At this temperature, metabolisms of both (phototropic) microeukaryotes and macrofauna are minimal (Lencioni 2004). Insufficient light to support photosynthesis could explain the depression of phototropic biomass (6-7% of total biomass) in winter, while lack of grazing pressure on heterotrophic prokaryotes could explain their absolute dominance of total microbial biomass during the coldest period of the year. With the onset of spring in March, the phototrophic eukaryotes increased in biomass, while microbial biomass reached periods of peak total biomass in late spring and summer (Table 1). In spring water has warmed sufficiently, mean monthly flow decreased and changes in light levels likely enhanced phototropic growth rates such that total microeukaryotic biomass accounted for 21% to 28% of total microbial biomass. However, increasing water temperature also increased macrofauna activities (Tande 1988) with resultant grazing pressure on microbial biomass as evidenced by the decline in total microbial biomass into the autumn.

Seasonal patterns in microbial community structure have previously been reported in several lotic environments (Smoot and Findlay 2001; Sekiguchi et al. 2002; Crump et al. 2003), and two studies described recurring seasonal patterns of microbial communities in streambed sediments through several annual cycles (Sutton and Findlay 2003; Hullar et al. 2006). The sediment microbial community composition of WCC displayed distinct seasonal patterns supporting earlier findings and paralleled the annual cycle in biomass loss and accrual. Our data showed that the seasonal pattern of variation was, in part, the result of shift between the ratios of prokaryotic to eukaryotic component of the community. This shift, quantified as PC1 score, was significantly correlated with seasonal changes in median daily flow, DOC concentration, high and
low flood pulse counts and water temperature. The correlation of variation in streamflow and temperature with changes in community structure of sediment communities observed in our study was consistent with other studies of temperate ecosystems that reported associated changes in productivity and community structure (Kaplan and Bott 1989; Shiah and Ducklow 1995; Sutton and Findlay 2003; Hullar et al. 2006). The negative effects of low temperature on microeukaryotic component of microbial community may be associated with decreased affinity for substrates or inability to sequester substrates from their environment at very low temperatures (Nedwell 1999). Also, variability in daily flow and high flood pulse count could reduce contact time with highly bioavailable DOC, which can alter microbial community structure and function. The positive effects of DOC on microbial community structure via changes in quantities and qualities of carbon availability have been documented to alter microbial community structure in predictable ways in both field and mesocosm studies (Falchini et al. 2003; Waldrop and Firestone 2004; Fierer et al. 2007; Nemergut et al. 2010).

The winter sediments (December - February) were dominated by prokaryotes; including functional groups of fatty acids indicating the presence of gram-positive, gram-negative, anaerobic, and sulfate-reducing bacteria. Earlier molecular assessments (Hullar et al. 2006) and a mesocosm study (Chapter 4) of WCC sediments showed that these fatty acids are indicative of a wide range of bacteria found in the α, β, γ and δ subclasses of proteobacteria and in several genera of Firmicutes, Acidobacteria, Bacteroidetes and Gammatimonadetes, which is consistent for other freshwater bacterioplankton communities (Zwart et al. 2002; Eiler and Bertilsson 2004). Storm events in winter accounted for over 30% (Table 1) of the yearly discharge, and consequently, a major driver that contributed to low microbial biomass through erosion and transport of streambed particles. Reductions in sediment bacterial biomass have been reported for
riverine sediments following storm events (Holmes et al. 1998; Eisenmann et al. 1999). However, benthic bacterial composition exhibited a lower disturbance threshold compared to eukaryotic biomass, which may reflect individual differences in attachment to streambed structure.

Microeukaryotic functional groups, including diatoms and other phototrophic microeukaryotes (as represented by 20:5w3, 18:3w3, and 16:4w1), at our site responded to the moderate warmer conditions in the spring and helped define microbial community structure. Dominant vernal photoautotrophs corresponding to *Haslea*, seasonally varying *Navicula* and cyanobacterial (*Phormidium subfuscum*) populations were previously determined in WCC based on a molecular assessment (Hullar et al. 2006). Kjeldsen et al. (1996) reported benthic algal spring development peaking in late April/mid May in a Danish lowland stream. This is in concert with Iversen et al. (1991) who reported the same pattern in a channelized stream. In several studies (Sand-Jensen et al. 1988; Horner et al. 1990; Kjeldsen et al. 1996), the initiation of spring blooms has been explained by increased irradiance and decreased water velocity and light attenuation. As streamwater continued to warm into the summer, studies of temperature-dependent metabolic activity suggests increases in macroinvertebrate activity (Tande 1988), thus, increased grazing pressure on phototrophs may have caused the decrease in phototrophic biomass observed in summer months (Table 1 and Fig. 2). This finding is in agreement with other studies showing that invertebrate grazing can regulate phototrophic biomass (Kohler 1992; Feminella and Hawkins 1995; Lambert 1996). Functional groups of fatty acids indicating the presence of fungi, protozoan, and bacteria progressively replaced the community and were more dominant in summer (Fig. 2). Saprophytic fungi and terrestrial plant detritus (plant root and shoot tissue) represented by 18:2w6 and 18:1w9 (Frostegård and Bååth, 1996 and Olsson and Johansen, 2000)
assumed dominance in the summer/late summer months, possibly due to an increased input of plant detritus towards the end of the growing season.

There were no seasonal differences in stable carbon isotope ratios for most bacterial PLFAs (e.g., i15:0, a15:0), but there were seasonal differences in summed feature 1 (16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t) and the fatty acid 20:5ω3. The fatty acids in summed feature 1 are all monounsaturated fatty acids and are found in a wide range of aerobic bacteria, and heterotrophic and phototrophic eukaryotes (Findlay 2004). The fatty acid 20:5ω3 is most often associated with phototrophic microeukaryotic (in particular, diatoms; Findlay 2004) and showed annual range in isotopic ratios of approximately -36‰ in spring (April) to -44‰ in Autumn (September) and Winter (December), indicating that isotopic fractionation was not constant seasonally (Ishikawa et al. 2012). Published values of carbon isotopic signatures for freshwater algae ranged between -47 and -12‰ (-25.7 ± 6.8, mean ± S.D.) (Finlay 2001; Zah et al. 2001; Ishikawa et al. 2012). Studies investigating factors that influence isotopic fractionation in phytoplankton have suggested that spatial and temporal variation in δ¹³C of CO₂ can play a major role in determining microalgal δ¹³C in streams (Findlay 2004). Since photosynthesis in spring is high and respiration on terrestrial organic matter is low, resulting in reduced input of CO₂ from decomposing ¹³C-depleted terrestrial detritus, the biological carbon fixation results in less negative δ¹³C for PLFA 20:5w3 (Golterman and Meyer 1985; Hellings et al. 2001). Moreover, some phototrophs such as diatoms can utilize bicarbonate, which is isotopically heavier than dissolved CO₂ (Law et al. 1995; Boschker et al. 2005; Ishikawa et al. 2012), thereby explaining the higher δ¹³C of 20:5w3 in spring. Overall, increased algal productivity in spring was partly due to moderate stream temperature and flow appears to be the strongest predictor of the variability in δ¹³C of PLFAs.
On average, the $\delta^{13}C$ values of many bacterial PLFAs were depleted by 2-5‰ relative to $\delta^{13}C$ of sediment total organic carbon, which was consistent with the findings of Canuel et al. (1997) that $\delta^{13}C$ values of lipids are generally depleted by 3-5‰ relative to $\delta^{13}C$ of sediment total organic carbon. This implied that bacteria were utilizing a carbon source with a $\delta^{13}C$ of -26 to -28‰, which is comparable to $\delta^{13}C$ of sediment total organic carbon in this study and a previous determination of the $\delta^{13}C$ of WWC DOC (-26.10; Wiegner et al, unpublished). The correspondence among the $\delta^{13}C$ of many bacterial PLFAs and sediment organic carbon and streamwater DOC supports previous studies concluding that bacterial carbon in forested streams is derived from streamwater DOC and particulate detritus (Bott et al. 1984; Hall 1995; Webster et al. 1999). These fatty acids were enriched in $^{13}C$, on average by 9-10‰ compared to 20:5w3. This difference support the utilization of terrigenous detrital carbon and suggests minimal utilization of autochthonous DOC. Boschker et al. (2005) reported uncoupled algal-bacterial system (also based on PLFA) with terrestrial organic matter or sewage as subsidies supporting bacteria growth during a spring bloom in the upper Scheldt estuarine. In contrast, $\delta^{13}C$ values of bacterial PLFA of summed feature 1 (16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t) showed enrichment in $\delta^{13}C$ by 5.77‰ relative to 20:5w3 suggesting that some heterotrophic prokaryotes within the system may utilize autochthonous DOC, along with allochthonous detritus as carbon sources. The observed $\delta^{13}C$ difference among microbial taxa suggested that further studies of carbon substrate dynamics in lotic systems are needed.

In conclusion, our results show an overall seasonality within freshwater sediment microbial communities and their associated lipid carbon isotope ratios. Bacterial lipids were isotopically depleted on average by 2‰ and 5‰ relative to $\delta^{13}C$ of total organic carbon and enriched relative to $\delta^{13}C$ algae-derived carbon source. In winter, the lowest $\delta^{13}C$ PLFA values
were observed likely due to enhanced input of CO$_2$ from detrital decomposition. During spring bloom, when litter input is lowest, photosynthesis is high, input of CO$_2$ from detrital decomposition is low resulting in less negative $\delta^{13}$C PLFAs. Thus, the annual variation of the $\delta^{13}$C of biota is sensitive in providing indication of changes resulting from biological carbon fixation and from degradation and respiration from aquatic biota or terrestrial detritus. As such, isotopic measurements may serve as an early warning signal of ecological changes related to ecological processes in natural ecosystems. Furthermore, these findings support an emerging picture in stream ecosystem that hydro-ecological parameters are important factors that structure streambed microbial communities. Seasonal changes in microbial community structure are largely predictable and allow the community to take advantage of the changes in carbon and energy input sources over an annual cycle. Several microbial biogeography studies now provide evidence for non-random patterns in microbial distribution and show that local environmental heterogeneity and geographical distance regulate microbial distribution (Battin et al. 2001; Oda et al. 2003; Crump et al. 2004; Hughes-Martiny et al. 2006; Fierer et al. 2007; Findlay et al. 2008). More studies and assessments of microbial seasonal patterns may help facilitate better monitoring strategies for detecting immediate as well as long-term impacts of anthropogenic stressors on the microbial communities of lotic ecosystems.

Acknowledgments

The authors wish to acknowledge Sherman Roberts for help obtaining samples, and Janna Brown for assistance in obtaining IRMS data. This research was supported, in part, by NSF grants DEB – DEB-0516235.


Figure 1. Mean daily discharge at United States Geological Survey (USGS) gauging station of the study stream during the study period from November 2009 to October 2010. Arrow represents sampling date of streambed sediment samples.
Figure 2. PCA of benthic microbial community structure determined by PLFA from the White Clay Creek seasonal sampling site. Scores are plotted by months: February, F; March, Ma; April, Ap; May, My; June, Ju; July, Jy; August, Au; September, S; October, O; December, D. Scales indicate the degree of difference among samples and influential fatty acids (factor loadings $>|0.5|$) are shown along each axis. Symbols indicate mean PC scores (n=9, except Nov. and Dec. where n =3), error bars = ±S.D. Ellipses drawn by hand to emphasize clusters.
Figure 3. Relationship between PCA factor 1 score and the calculated percentage that microeukaryotes contribute to total microbial biomass for all samples.
Figure 4. Seasonal variability in sedimentary TOC and selected $\delta^{13}$C PLFAs with component loadings $>0.5$ that exerted strong influence on the pattern of variation among samples along the PC 1 (Fig 5). Bars represent standard deviation.
Figure 5. PCA of all quantified $\delta^{13}$C of PLFAs of WCC benthic microbial community. Scores are plotted by months: February, FE; March, MA; April, AP; May, MY; June, JU; July, JY; September, SE; October, OC; December, DE. Influential fatty acids (factor loadings > $|0.5|\]) are shown along each axis. # summed feature includes 16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t; *summed feature includes 18:2ω6, 18:3ω3, 18:1ω9, 18:1ω7c, 18:1ω5
Table 1. Seasonal variations in total sedimentary microbial biomass, bacterial abundance, and physico-chemical parameters of White Clay Creek

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Biomass/PLP (nmol g⁻¹ dw)</th>
<th>Bacterial abundance (g⁻¹ dry wt)</th>
<th>Percent Eukaryotic/prokaryotic</th>
<th>Mean daily temperature (°C)</th>
<th>Mean Monthly Flow (m³ s⁻¹)</th>
<th>Mean DOC (µg/L)</th>
<th>Sediment δ¹³C</th>
<th>Sediment δ¹⁵N</th>
<th>Sediment %C</th>
<th>Sediment %N</th>
<th>C:N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>December</td>
<td>10.05 ± 6.95</td>
<td>3.78 x 10⁸</td>
<td>6/94</td>
<td>2.82</td>
<td>1.14</td>
<td>1260</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
</tr>
<tr>
<td>February</td>
<td>10.64 ± 3.67</td>
<td>3.96 x 10⁸</td>
<td>7/93</td>
<td>3.82</td>
<td>0.61</td>
<td>956</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
</tr>
<tr>
<td>March</td>
<td>18.34 ± 8.47</td>
<td>5.66 x 10⁸</td>
<td>23/77</td>
<td>7.56</td>
<td>1.22</td>
<td>1632</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
</tr>
<tr>
<td>April</td>
<td>16.23 ± 3.29</td>
<td>5.33 x 10⁸</td>
<td>18/82</td>
<td>12.31</td>
<td>0.72</td>
<td>1199</td>
<td>-27.22 ± 0.70</td>
<td>2.87 ± 1.75</td>
<td>1.20 ± 0.35</td>
<td>0.11 ± 0.03</td>
<td>10.79</td>
</tr>
<tr>
<td>May</td>
<td>27.19 ± 9.00</td>
<td>7.80 x 10⁸</td>
<td>28/72</td>
<td>16.13</td>
<td>0.46</td>
<td>1470</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
<td>LIT</td>
</tr>
<tr>
<td>June</td>
<td>28.96 ± 12.13</td>
<td>9.19 x 10⁸</td>
<td>21/79</td>
<td>16.64</td>
<td>0.27</td>
<td>1274</td>
<td>-26.69 ± 0.64</td>
<td>-0.81 ± 2.70</td>
<td>0.95 ± 0.52</td>
<td>0.06 ± 0.03</td>
<td>14.74</td>
</tr>
<tr>
<td>July</td>
<td>19.91 ± 11.95</td>
<td>7.10 x 10⁸</td>
<td>11/89</td>
<td>19.83</td>
<td>0.32</td>
<td>1757</td>
<td>-26.09 ± 1.27</td>
<td>-5.45 ± 4.83</td>
<td>0.57 ± 0.53</td>
<td>0.06 ± 0.04</td>
<td>9.92</td>
</tr>
<tr>
<td>August</td>
<td>13.94 ± 1.45</td>
<td>3.90 x 10⁶</td>
<td>30/70</td>
<td>20.33</td>
<td>0.16</td>
<td>1932</td>
<td>-25.27 ± 0.25</td>
<td>2.00 ± 1.15</td>
<td>0.26 ± 0.02</td>
<td>0.02 ± 0.00</td>
<td>13.02</td>
</tr>
<tr>
<td>September</td>
<td>13.35 ± 1.29</td>
<td>4.14 x 10⁶</td>
<td>23/77</td>
<td>14.3</td>
<td>0.15</td>
<td>1357</td>
<td>-27.17 ± 0.85</td>
<td>2.77 ± 0.49</td>
<td>0.49 ± 0.23</td>
<td>0.04 ± 0.03</td>
<td>12.66</td>
</tr>
<tr>
<td>October</td>
<td>16.92 ± 6.76</td>
<td>4.93 x 10⁸</td>
<td>27/73</td>
<td>11.82</td>
<td>0.55</td>
<td>2550</td>
<td>-26.55 ± 1.78</td>
<td>2.96 ± 0.77</td>
<td>0.43 ± 0.26</td>
<td>0.03 ± 0.02</td>
<td>12.77</td>
</tr>
</tbody>
</table>

LIT = Lost in transit
Table 2. Pearson correlation coefficient matrices between selected hydrological indices and measured environmental variables.

<table>
<thead>
<tr>
<th></th>
<th>Biomass</th>
<th>Tempt (°C)</th>
<th>DOC (ug/L)</th>
<th>MDF</th>
<th>MQ50</th>
<th>MVD1</th>
<th>MBI</th>
<th>FLC</th>
<th>FHC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>0.519</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOC (ug/L)</td>
<td>0.078</td>
<td>0.385</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDF</td>
<td>-0.320</td>
<td>-0.702</td>
<td>-0.389</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MQ50</td>
<td>-0.066</td>
<td>-0.510</td>
<td>-0.276</td>
<td>0.887</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVD1</td>
<td>-0.552</td>
<td>-0.465</td>
<td>-0.283</td>
<td>0.519</td>
<td>0.093</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBI</td>
<td>0.488</td>
<td>0.350</td>
<td>0.181</td>
<td>-0.527</td>
<td>-0.134</td>
<td>-0.974</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLC</td>
<td>-0.192</td>
<td>-0.220</td>
<td>-0.532</td>
<td>0.549</td>
<td>0.342</td>
<td>0.650</td>
<td>-0.706</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>FHC1</td>
<td>-0.628</td>
<td>-0.534</td>
<td>-0.122</td>
<td>0.365</td>
<td>0.204</td>
<td>0.401</td>
<td>-0.382</td>
<td>0.477</td>
<td>1</td>
</tr>
</tbody>
</table>

Bold mean r statistics for variable > |0.7|
Italic means variables removed prior to ‘best subset’ multiple regression analysis
Table 3. Multiple regression analysis (best subsets) for natural log biomass as a function of stream physico-chemical and hydrological indices

<table>
<thead>
<tr>
<th>Model</th>
<th>Vars</th>
<th>R-Sq</th>
<th>R-Sq(adj)</th>
<th>Mallow Cp</th>
<th>SE</th>
<th>Temp</th>
<th>DOC</th>
<th>MQ50</th>
<th>MVD1</th>
<th>FLC</th>
<th>FHC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>39.4</td>
<td>32.7</td>
<td>0.8</td>
<td>4.9865</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>30.5</td>
<td>22.8</td>
<td>1.9</td>
<td>5.3405</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>50.2</td>
<td>37.7</td>
<td>1.4</td>
<td>4.7965</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>44.1</td>
<td>30.2</td>
<td>2.2</td>
<td>5.0783</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>64.3</td>
<td>49.0</td>
<td>1.6</td>
<td>4.3381</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>51.5</td>
<td>30.8</td>
<td>3.2</td>
<td>5.0561</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>65.7</td>
<td>42.8</td>
<td>3.4</td>
<td>4.5952</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>64.7</td>
<td>41.2</td>
<td>3.5</td>
<td>4.6588</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>66.2</td>
<td>32.5</td>
<td>5.3</td>
<td>4.9926</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>65.9</td>
<td>31.9</td>
<td>5.4</td>
<td>5.0151</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>68.8</td>
<td>22.1</td>
<td>7.0</td>
<td>5.3628</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

93
Table 4. Multiple regression analysis (best subsets) for microbial community (PC1) as a function of stream physico-chemical and hydrological indices

<table>
<thead>
<tr>
<th>Model</th>
<th>Vars</th>
<th>R-Sq</th>
<th>R-Sq (adj)</th>
<th>Mallow Cp</th>
<th>SE</th>
<th>% Eukaryotic</th>
<th>Temp</th>
<th>DOC</th>
<th>MQ50</th>
<th>MVD1</th>
<th>FLC</th>
<th>FHC1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>57.1</td>
<td>51.8</td>
<td>28.1</td>
<td>0.62343</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>31.0</td>
<td>22.4</td>
<td>48.8</td>
<td>0.79067</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>78.3</td>
<td>72.1</td>
<td>13.3</td>
<td>0.47435</td>
<td>X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>65.1</td>
<td>55.2</td>
<td>23.7</td>
<td>0.60108</td>
<td>X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>85.6</td>
<td>78.3</td>
<td>9.5</td>
<td>0.41777</td>
<td>X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>84.3</td>
<td>76.4</td>
<td>10.5</td>
<td>0.43577</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>90.4</td>
<td>82.8</td>
<td>7.6</td>
<td>0.37224</td>
<td>X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>88.7</td>
<td>79.7</td>
<td>9.0</td>
<td>0.40484</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>97.0</td>
<td>93.2</td>
<td>4.4</td>
<td>0.23342</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>92.8</td>
<td>83.7</td>
<td>7.8</td>
<td>0.36226</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>97.4</td>
<td>92.2</td>
<td>6.1</td>
<td>0.25024</td>
<td>X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>97.1</td>
<td>91.4</td>
<td>6.3</td>
<td>0.26313</td>
<td>X X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>97.5</td>
<td>88.7</td>
<td>8.0</td>
<td>0.30210</td>
<td>X X X X X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. Annual variations in stable carbon isotope signatures for selected common fatty acids from White Clay Creek sediments.

Values shown are mean (±SD) from all sampling sites and months.

<table>
<thead>
<tr>
<th>FAMEs</th>
<th>FEB</th>
<th>MAR</th>
<th>APR</th>
<th>MAY</th>
<th>JUN</th>
<th>JUL</th>
<th>SEP</th>
<th>OCT</th>
<th>DEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>-37.64 ±1.53</td>
<td>-37.24 ±2.75</td>
<td>-32.03 ±1.60</td>
<td>-35.69 ±0.67</td>
<td>-36.34 ±1.01</td>
<td>-34.56 ±1.97</td>
<td>-37.47 ±1.55</td>
<td>-34.50 ±1.30</td>
<td>-34.98 ±1.00</td>
</tr>
<tr>
<td>i15:0</td>
<td>-30.36 ±0.83</td>
<td>-30.19 ±0.51</td>
<td>-29.61 ±0.46</td>
<td>-30.53 ±0.55</td>
<td>-31.20 ±0.63</td>
<td>-31.59 ±1.41</td>
<td>-31.74 ±0.70</td>
<td>-30.69 ±0.56</td>
<td>-29.70 ±0.94</td>
</tr>
<tr>
<td>a15:0</td>
<td>-28.95 ±1.72</td>
<td>-29.72 ±0.86</td>
<td>-29.34 ±0.63</td>
<td>-29.20 ±0.91</td>
<td>-30.24 ±0.53</td>
<td>-30.41 ±0.72</td>
<td>-30.78 ±0.54</td>
<td>-29.97 ±0.60</td>
<td>-29.32 ±0.91</td>
</tr>
<tr>
<td>15:0</td>
<td>-30.97 ±2.71</td>
<td>-35.34 ±0.90</td>
<td>-30.66 ±2.08</td>
<td>-30.73 ±1.82</td>
<td>-32.11 ±1.50</td>
<td>-29.53 ±0.50</td>
<td>-32.86 ±1.67</td>
<td>-30.00 ±0.84</td>
<td>ND</td>
</tr>
<tr>
<td>i16:0+15:1</td>
<td>-28.23 ±1.45</td>
<td>-29.53 ±0.75</td>
<td>-29.10 ±0.59</td>
<td>-28.36 ±1.25</td>
<td>-30.44 ±0.51</td>
<td>-31.94 ±1.33</td>
<td>-31.14 ±0.62</td>
<td>-29.63 ±0.79</td>
<td>-28.43 ±1.00</td>
</tr>
<tr>
<td>16:0</td>
<td>-32.65 ±2.24</td>
<td>-34.32 ±1.21</td>
<td>-31.90 ±2.05</td>
<td>-34.45 ±0.57</td>
<td>-32.92 ±2.53</td>
<td>-34.28 ±1.98</td>
<td>-35.20 ±0.84</td>
<td>-34.68 ±2.24</td>
<td>-33.79 ±1.65</td>
</tr>
<tr>
<td>SF1</td>
<td>-32.58 ±1.33</td>
<td>-33.91 ±1.31</td>
<td>-31.70 ±1.71</td>
<td>-34.43 ±0.79</td>
<td>-30.99 ±2.67</td>
<td>-35.32 ±1.00</td>
<td>-35.47 ±1.17</td>
<td>-35.26 ±1.76</td>
<td>-36.08 ±1.27</td>
</tr>
<tr>
<td>cy17:0</td>
<td>-32.49 ±4.80</td>
<td>-30.04 ±0.64</td>
<td>-30.16 ±0.68</td>
<td>-31.30 ±0.80</td>
<td>-30.28 ±1.15</td>
<td>-32.56 ±1.06</td>
<td>-31.50 ±0.90</td>
<td>-32.14 ±2.39</td>
<td>-30.78 ±1.73</td>
</tr>
<tr>
<td>18:0</td>
<td>-30.82 ±0.32</td>
<td>-31.91 ±0.94</td>
<td>-30.64 ±0.29</td>
<td>-32.97 ±0.82</td>
<td>-32.28 ±2.02</td>
<td>-33.54 ±1.23</td>
<td>-33.81 ±1.14</td>
<td>-32.97 ±1.57</td>
<td>-31.27 ±0.14</td>
</tr>
<tr>
<td>SF2</td>
<td>-30.74 ±1.46</td>
<td>-32.03 ±0.69</td>
<td>-31.26 ±0.38</td>
<td>-31.85 ±0.55</td>
<td>-31.18 ±1.13</td>
<td>-32.35 ±0.45</td>
<td>-34.23 ±0.53</td>
<td>-33.00 ±1.71</td>
<td>-31.94 ±0.17</td>
</tr>
<tr>
<td>18:2w6</td>
<td>-33.43 ±3.31</td>
<td>-37.64 ±2.32</td>
<td>-32.63 ±1.44</td>
<td>-36.16 ±1.08</td>
<td>-36.05 ±3.06</td>
<td>-37.77 ±2.06</td>
<td>-38.32 ±2.70</td>
<td>-38.22 ±4.70</td>
<td>-38.94 ±1.00</td>
</tr>
<tr>
<td>18:3w3</td>
<td>-32.10 ±0.73</td>
<td>-32.37 ±0.65</td>
<td>-32.31 ±0.53</td>
<td>-33.10 ±0.36</td>
<td>-33.12 ±0.58</td>
<td>-34.62 ±0.79</td>
<td>-33.58 ±0.42</td>
<td>-34.37 ±1.71</td>
<td>-32.05 ±0.33</td>
</tr>
<tr>
<td>20:0</td>
<td>-36.60 ±1.62</td>
<td>-42.52 ±1.78</td>
<td>-38.57 ±0.95</td>
<td>-40.78 ±1.73</td>
<td>-41.68 ±1.49</td>
<td>-42.06 ±2.05</td>
<td>-42.12 ±2.42</td>
<td>-41.90 ±3.69</td>
<td>ND</td>
</tr>
<tr>
<td>20:4w6</td>
<td>-37.60 ±3.25</td>
<td>-40.32 ±1.78</td>
<td>-36.08 ±0.87</td>
<td>-38.46 ±1.33</td>
<td>-40.94 ±0.86</td>
<td>-38.92 ±1.24</td>
<td>-41.50 ±2.01</td>
<td>-39.83 ±2.24</td>
<td>ND</td>
</tr>
<tr>
<td>20:5w3</td>
<td>-41.18 ±1.75</td>
<td>-38.90 ±1.34</td>
<td>-36.17 ±2.77</td>
<td>-39.61 ±0.75</td>
<td>-42.56 ±0.88</td>
<td>-40.36 ±1.73</td>
<td>-43.66 ±1.41</td>
<td>-39.23 ±2.32</td>
<td>-43.89 ±2.03</td>
</tr>
</tbody>
</table>

SF1 = summed feature including 16:1ω9, 16:1ω7c, 16:1ω5c, 16:1w13t; SF2 = summed feature including 18:1ω9, 18:1ω7c, 18:1ω5.
ND = not detected
CHAPTER 4

ELUCIDATING THE BACTERIA RESPONSIBLE FOR UTILIZATION OF DISSOLVED ORGANIC MATTER IN A THIRD-ORDER STREAM

ABSTRACT

Terrigenous dissolved organic matter (DOM) has long been considered recalcitrant to bacterial biodegradation although current research has shown its susceptibility to microbiological and photolytic oxidations and that it contributes significantly to the energy flow in aquatic ecosystems. To determine the microbial groups actively utilizing terrestrially derived streamwater DOM, we characterized sediment microbial biomass and community structure using phospholipid phosphate and phospholipid fatty acids analysis and identified metabolically active members using phospholipid fatty acid stable isotope probing. Prokaryotes comprised 61% of the streambed microbial community and consisted of aerobic, facultative anaerobic and anaerobic bacteria while microeukaryotes comprised the remaining 39%. Streambed sediments were incubated in re-circulating mesocosm chambers amended with leachate from composted $^{13}$C-labelled tulip poplar tree-tissues (a process that yields $^{13}$C-labeled DOM with size and lability fractions approximating streamwater DOM) and examined for $^{13}$C incorporation into microbial phospholipid fatty acids (PLFAs). The structure of stream sediment microbial communities prior to and after mesocosm incubation, in both the presence and absence of $^{13}$C-labeled DOM, showed no significant differences and indicated our mesocosm-based experimental design as sufficiently
robust to investigate the utilization of $^{13}$C-DOM by sediment microbial communities. After 48 hours of incubation, bacterial fatty acids i15:0, a15:0, 16:0, 16:1ω9, 18:1ω9c, 18:1ω7c, 10me16 and cy19:0 showed increased abundance of $^{13}$C. This identified the aerobic, facultative anaerobic and anaerobic bacteria as actively utilizing the $^{13}$C-labeled DOM. A single dark 48 h incubation showed incorporation into both bacterial and microeukaryotic fatty acids (20:4ω6, 20:5ω3) suggesting that the microeukaryotic predators consumed bacteria that utilized $^{13}$C-labeled DOM. Our data support the hypothesis that streamwater DOM is utilized by stream bacteria, and substantially contributes to the energy flow in aquatic ecosystems.
INTRODUCTION

Dissolved organic matter (DOM) plays a significant metabolic role in aquatic ecosystems as carbon and energy sources for the microbial food web (Peduzzi et al. 2008; Wiegner et al. 2009; Wong and Williams 2010). It influences the availability of dissolved nutrient and metals, and modifies the optical properties of aquatic ecosystems (Findlay and Sinsabaugh 1999; Sulzberger and Durisch-Kaiser 2009). In addition, DOM is now seen as an important driver of ecosystem functions in freshwater environments and a major component in global carbon cycling and climate change (Amon and Benner 1996; Batin et al. 2008; Besemer et al. 2009). Dissolved organic carbon (DOC) is the largest pool of organic carbon in aquatic ecosystems, is heterogeneous in nature being comprised of humic high-molecular-weight (HMW >1 kDa) and non-humic low-molecular-weight (LMW <1 kDa) fractions (Amon and Benner 1996; Rosenstock et al. 2005) and may contain upwards of 5500 individual organic compounds between 300 and 1 kDa (Mosher et al. 2010). This complex mixture is present in all natural waters and is continuously supplied to aquatic ecosystems from both allochthonous (terrestrial) and autochthonous (aquatic) sources (Peduzzi et al. 2008). Amon and Benner (1996) reported that humic substances of terrestrial origin are the major constituents of the DOC pool in stream ecosystems and comprise up to 88% of the DOC in the high molecular weight fraction in the Amazon River water. These findings agree with other studies, which found that, in most cases, terrigenous DOC comprised a large portion of DOM in streams and rivers (Benner and Hedges 1993; Hedges et al. 1994; Peduzzi et al. 2008). Conventionally, terrigenous DOC has been considered recalcitrant to bacterial biodegradation and to move conservatively through aquatic ecosystems due to the apparent biochemical refractory nature of humic substances (Mantoura and
Woodward 1983; Thurman 1986; Rosenstock et al. 2005). However, Volk et al. (1997) found that humic substances account for 75% of the biodegradable fraction of DOM in White Clay Creek. Similar studies confirm the susceptibility of terrigenous DOC to microbiological and photolytic oxidations, as well as their value as microbial substrates and their significant contribution to energy flow in aquatic ecosystems (Amon and Benner 1996; Bano et al. 1997; Carlsson et al. 1999; Frazier et al. 2005; Kaplan et al. 2008; Battin et al. 2008; Fagerberg et al. 2009).

Heterotrophic benthic bacteria are important organisms in lotic ecosystems and are responsible for several biogeochemical transformations, including DOC uptake, degradation, and mineralization (Kaplan and Newbold 1993; Pusch et al. 1998; Fischer and Pusch 2001; Tank et al. 2010). While it is clearly established that bacteria provide an important trophic linkage between DOM and many stream fauna (Hall and Meyer 1998), their relative importance in overall stream carbon processing remains relatively understudied (Tank et al. 2010). In particular, little is known about which heterotrophic benthic bacteria drive these ecosystem dynamics. Moreover, research efforts to understand DOM utilization through microbial processes have been complicated by the chemical heterogeneity of the DOM pool (Mosher et al. 2010) and a lack of methods for measuring in situ microbial activities (Kaplan et al. 2008; Bourguet et al. 2009). Few studies have attempted to identify substrates that would be representative of natural molecules providing realistic data regarding DOM utilization. Such attempts include NaH\(^{13}\)CO\(_3\) additions in lakes (Kritzberg et al. 2004; Pace et al. 2004) and \(^{13}\)C-enriched sodium acetate additions in streams (Hall and Meyer, 1998; Johnson and Tank 2009). However, being chemically much simpler than terrestrially derived DOM, these tracers are not reflective of natural stream water DOM. This major drawback has been addressed by the production of a terrestrial DOC tracer; \(^{13}\)C-labeled tree tissue leachate from tulip poplar tree leaves, small twigs and roots with polymeric
and monomeric constituents and lability fractions approximating those of stream water DOC (Wiegner et al. 2005a). Using this $^{13}$C-DOC tracer, Wiegner et al. (2005b) reported that labile DOM is taken up quickly at or near its point of entry to the stream, whereas intermediately labile /humic DOM is consumed more slowly and a substantial proportion is exported to downstream reaches, thus humic DOM serves as an important energy link between upstream and downstream systems (Kaplan et al. 2008). These results indicated that $^{13}$C-labeled tree tissues leachate is the most representative tracer of allochtonous DOC inputs to streams that has been used to date and is well suited to investigate the utilization of DOC by heterotrophic benthic microbes.

The goal of our study was to elucidate the heterotrophic benthic microbes within stream sediments that actively utilize terrestrial DOC, thereby controlling C flux to higher trophic levels and to downstream reaches. We examined incorporation of terrestrial DOC into microbial biomass by incubating stream sediment in recirculating mesocosms with natural stream water to which tracer-levels of $^{13}$C-labeled tree tissues leachate were added. We used phospholipid fatty acid (PLFA) analysis to characterize the benthic microbial biomass and community structure (Findlay 2004) and PLFA stable isotope probing (SIP) to determine the metabolically active community members (Boschker et al. 1998; Boschker 2004). We have extended the specificity of functional assignments of PLFA-SIP by utilizing clone libraries produced from White Clay Creek sediments by Hullar et al. (2006) and published phenotypic descriptions of identified species (e.g., Hahn et al. 2010, Jin et al., 2012).
MATERIALS AND METHODS

Study site

Streamwater and sediments were collected from 3rd order White Clay Creek (WCC) adjacent to the Stroud Water Research Center in Avondale, Pennsylvania, and two 2nd order streams; the West (WCCW) and East (WCCE) branches of White Clay Creek. The White Clay Creek watershed is agriculturally dominated with upstream riparian forests and is within the Piedmont Province of southeastern Pennsylvania and northern Delaware (39°53’ N, 75°47’ W), joining the Christina River near the Christina’s discharge to the Delaware Bay. White Clay Creek drains 725 ha of approximately 52% of agricultural, 22% of tilled/hayed and 23% of wooded lands (Newbold et al. 1997; Wiegner et al. 2005b). The immediate area surrounding the three study sites is forested and the local drainage is a patchwork of pasture lands grazed by horses and cattle. The dominant tree species reported are tulip poplar (Liriodendron tulipefera), beech (Fagus grandifolia), red oak (Quercus rubra), and black oak (Quercus velutina) (Wiegner et al. 2005b). Streamflow and streamwater chemistry has been monitored at regular intervals since the 1970s with mean annual stream flow, stream water temperature, and local precipitation of 115 L/s, 10.6°C, and 105 cm y⁻¹, respectively (Newbold et al. 1997). Streambed sediments consist of clay-, silt-, and sand-sized particles in pools and runs, with gneiss- and schist-derived gravel and cobble in riffles (Kaplan et al. 1980).
Synthesis of $^{13}$C-labeled DOM

Wiegner et al. (2005a) describes the generation of the $^{13}$C-labeled stream DOM. Briefly, thirty-two 1-y-old tulip poplar seedlings (Liriodendron tulipefera L.) were grown with $^{13}$CO$_2$ at the National Phytotron located at Duke University, Durham, North Carolina, USA. $^{13}$C-tree tissue leachate was generated by leaching approximately 4 g of dried, ground tulip poplar seedlings tissues (60.9% leaves, 24.6% stems, 14.5% roots; % weight of new tissues) in 4 L of sterile-filtered (0.2-µm, Gelman Supor) C-free de-ionized cold water in the dark at 4° C for 24 h. The mixture of all tree tissue types (leaf, stem, and root) was used to generate a leachate representative of fresh tree litter inputs (organic matter inputs) from trees to streams. Tree tissue leachate was Tyndallized in a 70 °C water bath for 0.5 h twice, separated by 24 h at room temperature to ensure biological stability, and stored in 2-L sterile plastic containers in the dark at 4 °C (Wiegner et al. 2005a) until the experiment began.

Mesocosms

Two 15-L recirculating plug-flow bioreactors with an empty bed contact time of 150 min were used to elucidate the microbes responsible for utilization of DOM in streams. Bioreactors accepted a 0.014 m$^2$ galvanized box used to sample stream sediments such that the stream sediments were level with the bioreactor bed. Bioreactors could be operated in either open or recirculating mode and were contained within a 1000 L flowing stream water tank to maintain ambient stream temperature. These tanks also served as the source of streamwater during open mode operation. Sondes with dissolved O$_2$ and temperature/conductivity probes (YSI Model 600 XL, Yellow Springs, Inc., Yellow Springs, Ohio) were inserted into the recirculation line of each
mesocosm. Mesocosms were set up in the experimental greenhouse facility of Stroud Water Research Centre under natural photoperiod except for experiment 1 (see below).

Sediment Collection and Mesocosm Experiments

Streambed sediments were collected in 2009 and 2010; four samples were collected at each sampling period. Sediments were collected from White Clay Creek in October 2009 (twice), April 2010 and August 2010, and from the west and east branches in November 2009. To collect stream sediments with a minimum of disturbance, a galvanized box (surface area 0.014 m$^2$) perforated by 0.32-cm diameter holes (bottom only) that allowed streamwater to escape as the box was inserted into the sediments until the bottom just touched the sediment surface. Plexiglas plates were slipped under and over the box trapping the sediments and allowed them to be lifted from the stream intact. A second box, just large enough to accommodate the first, was placed over the first box, the core inverted and the inner (first) box removed. This process yielded a rectangular core or sample of intact stream sediment that was placed into a bioreactor in the proper vertical orientation. Two samples were processed immediately and served as a reference for sediment and microbial community characteristics before the $^{13}$C-DOM uptake experiment (stream control; referred to as T0). The other two boxes were placed into 15-L recirculating bioreactors (one per bioreactor), which were operated for 24 h in open mode with a flow of 0.06 cm/s (equivalent to measured stream velocities). The chambers were switch to recirculate mode and one of the chambers (experimental; referred to as T$^{13}$C) received $^{13}$C DOM while the other chamber (mesocosm control; referred to as TM) received no $^{13}$C DOM.
Addition of the tracer increased total DOM by approximately 1-5%. Sediments were incubated in recirculation mode for 48h or 50h; $^{13}$C exposure period was based upon the effective depth of the chambers (ratio of chamber volume to sediment tray area) and the mass transfer coefficient for the $^{13}$C tracer (estimated from a prior whole-stream injection). At the end of the incubation period, the core was removed from the bioreactor, the upper 2 mm of sediments were removed with a clean spatula, placed in an aluminum weigh boat, well mixed, and subsampled for total microbial biomass, microbial community structure, $\delta^{13}$C of PLFAs, organic matter contents and particle-size analyses. In total six mesocosms experiments were conducted over the two-year period.

Experiment 1 used White Clay Creek sediments and the experiment was run in the dark for 48 h; the mesocosm chambers were covered with 2.2 cm Styrofoam sheets and black plastic sheeting to exclude light (Table 1). Experiment 2 was a replicate of experiment 1, except sediments were incubated uncovered (exposed to natural sunlight at natural photoperiod) and for 50 hours.

Experiments 3 and 4 were replicates of experiment 2 except sediments from west and east branches of WCC, respectively, were used. Experiment 5 and 6 replicated experiment 2. The concentrations of the $^{13}$C- tree tissue leachate used and exposure time are shown in Table 1. In August 2010, sediments were also collected from White Clay Creek at the end (T72) of the mesocosm incubations to assess natural changes in the microbial community over the period of the mesocosm experiment.

Phospholipid fatty acids analysis

Samples for lipid analysis were stored at -80°C until lyophilized. Microbial biomass and community structure of freeze-dried sediments (approximately 10 g dry weight) were determined using phospholipid analyses following the methods of Findlay (2004). Briefly, stream and
Mesocosm sediments were extracted in the dark at 4°C in 50ml screw-cap glass tubes with 27ml of a 1:2:0.6 (v/v/v) dichloromethane-methanol-50 mM phosphate buffer (pH 7.4) solution. The solution was partitioned into organic and aqueous phases with 7.5ml dichloromethane and 7.5ml deionized water, after which the organic phase (containing total lipid) was collected through a dry 2V filter (Whatman, Schleicher & Schuell) into 15ml test tubes and the solvent dried under nitrogen at 37°C. The dried lipid was dissolved in 2ml chloroform and two 100µl subsamples were oxidized with potassium persulfate at 100 °C overnight in sealed ampoules to release orthophosphate. Phosphate content was determined spectrophotometrically (610nm) using a dye-coupled reaction between ammonium molybdate and malachite green. The remainder of the lipid was fractionated into neutral, glyco-, and phospholipid with silica gel solid phase extraction chromatography. Phospholipid fatty acids were converted into their respectively methyl esters by base methanolation and purified by octadecyl bonded silica gel (C18) reverse-phase column chromatography. Purified fatty acid methyl esters (FAMEs) were identified and quantified using Agilent gas chromatograph equipped with an automatic sampler, a 60m x 0.25 mm non polar DB-1 column and a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 2.3ml/min. The initial chromatograph oven temperature was 80°C followed by a temperature rise of 4 °C/min to 250 °C which was then held at this temperature for 10min. FAME identification was based on relative retention times, coelution with standards, and mass spectral analysis.

Standard nomenclature was used to refer to the fatty acids: the total number of carbon atoms is followed by a colon, and the number of double bonds. The position of the first double bond is indicated by ω and the number of carbon atoms from the aliphatic end. For example, the fatty acid 16:1ω7, is 16 carbons long, and has one double bond that occurs at the seventh carbon from the omega end of the molecule. All double bounds are cis, unless designated as trans configuration.
using a suffix of $t$. Methyl branching at the *iso* and *anteiso* positions and at the 10th carbon atom from the carboxyl end is designated by the prefixes *i*, *a*, and 10Me, respectively. The prefix *cy* denotes cyclopropane fatty acids. Individual fatty acids were analyzed for both absolute and relative abundance. Relative abundance or weight percent data (gram individual fatty acids x gram$^{-1}$ total fatty acids x 100) was used to determine community structure (Findlay and Dobbs 1993). In addition to the standard combination of functional group and marker fatty acid assignments (Findlay 2004), we increased the specificity of PLFA taxonomic assignments using a previously published 16S rRNA gene library constructed from WCC sediments (Hullar et al. 2006) and the taxonomic descriptions of these OTUs or closely related species.

**Microbial community utilization of $^{13}$C DOM**

Stable carbon isotope ratios of individual FAMEs were determined using an Agilent 6890 GC coupled to a PRISM (GV Instruments, Manchester, UK) stable isotope mass spectrometer. Analyses were conducted on two different gas chromatographic columns (DB-1 and DB-23; 60m x 0.25mm, 0.25µm film thickness) to allow the analysis of a greater number of resolved fatty acid methyl esters. For FAMEs that were not resolved by either column during GC-IRMS analysis, we report the $\delta^{13}$C of the summed feature. Stable carbon isotope ratios were expressed as:

$$\delta^{13}\text{C} = [(R_{\text{sample}} / R_{\text{standard}}) -1] \times 1000 \quad (1)$$

where R is $^{13}$C/$^{12}$C in the samples and standard. Data were reported relative to Vienna PeeDee-Belemnite (VPDB). Incorporation of $^{13}$C into PLFAs was estimated using the equations (Abraham et al. 1998):

$$\delta^{13}\text{C}_{\text{FA}} = [(C_n + 1) \times \delta^{13}\text{C}_{\text{FAME}} - \delta^{13}\text{C}_{\text{MeOH}}] / C \quad (2)$$
where $\delta^{13}C_{FA}$ is the $\delta^{13}C$ of the fatty acid, $C_n$ is the number of carbons in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}C$ of the fatty acid methyl ester (FAME), and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol used for the methylation reaction, which was determined to be -43.23‰.

**Statistical analysis**

We used comparisons of total microbial biomass and community structure to assess the efficacy of our mesocosm approach with a comparison of stream sediments (T0) to the control mesocosm sediments (TM) used to assess mesocosm effects and comparison of control mesocosm sediments to treatment mesocosm sediments (T$^{13}C$) used to check for unwanted stimulation due to tracer-level DOM additions. Potential differences in microbial biomass and percent eukaryotes vs. prokarotes were examined with matched pairs t-tests with a $\alpha$-level of 0.05 using SPSS 19. Fatty acid profiles for the bioreactors and WCC sediments were subjected to principal component analysis (PCA) after log transformation [ln (x + 1)] of weight percent fatty acid data and analyzed using SPSS 19. Changes in microbial community structure were examined by comparing principal component 1 scores as above. Increases in $\delta^{13}C$ values of individual fatty acids, or when necessary summed features, upon exposure to $\delta^{13}C$-DOM were detected using a matched pairs t-tests of the difference in $\delta^{13}C$ values from control and treatment mesocosm sediments.
RESULTS

Utilization of $^{13}$C-labeled DOM by sediment microbial community

The combined DB-1 and DB-23 columns allowed the quantification of the $\delta^{13}$C values of 15 features; these were either individual PLFAs, two co-eluting PLFAs or summed features (more than 2 co-eluting PLFAs). For PLFAs that were resolved by both columns, values presented are the mean of both analyses (Table 2). PLFA $\delta^{13}$C values for stream and bioreactor control sediments (treatments T0 and TM) ranged from -37.26 to -28.53‰ and -35.92 to -28.70‰, respectively, with the most depleted values found in the microeukaryotic biomarkers 20:4$\omega_6$ and 20:5$\omega_3$, and the most enriched values found in cy17:0 (Table 2). No significant differences were observed between the $\delta^{13}$C values of PLFAs from T0 and TM treatments. The $\delta^{13}$C values of PLFAs from the $^{13}$C-labeled bioreactor ($T^{13}$C) ranged from -34.75 to -24.69‰. Eight features (5 individual PLFAs, two co-eluting pairs and 1 summed feature) showed significant $^{13}$C enrichment ($p<0.05$); these were i15:0, a15:0, 16:0, 10me16:0, cy19:0, 16:0/16:1$\omega_9$, 16:0$\omega_9$/18:1$\omega_7$ and summed feature 2 (18:2$\omega_6$, 18:3$\omega_3$, 18:1$\omega_9$, 18:1$\omega_7c$, 18:1$\omega_5$). The PLFA 18:2$\omega_6$ and 18:3$\omega_3$ were resolved by the DB-23 and did not show significant enrichments in $T^{13}$C, suggesting that significant enrichment in summed feature 2 detected using the DB-1 column was driven by enrichment of 18:1$\omega_9$/18:1$\omega_7$. Two polyenoic fatty acids, 20:5$\omega_3$ and 20:4$\omega_6$, indicative of microeukaryotes were significantly labeled in the dark-incubated bioreactor with a 4.15‰ and 3.45‰ differences (TM vs $T^{13}$C) respectively, while labeling of these fatty acids was not detected when incubations were conducted using the natural photoperiod. Assigning microbial identity to the enriched PLFAs using functional group approach (Findlay 2004), bacteria actively metabolizing stream water DOM were aerobic gram-negative bacteria (16:0/16:1$\omega_9$, ...)
18:1ω9/18:1ω7), gram-positive or facultative anaerobic gram-negative bacteria (i15:0, a15:0), and anaerobic gram-negative bacteria (10Me16:0, cy19:0).

Hullar et al. (2006) produced bacterial 16S rRNA gene clone libraries from White Clay Creek sediment (same site as used in this study). Using the highest available taxonomic resolution (species, genus, or phylum) and published phenotypic descriptions, we matched bacterial taxa known to inhabit White Clay Creek to the PLFAs showing $^{13}$C enrichment during incubation with $^{13}$C-labeled DOM (Table 3). The fatty acid i15:0 is a major fatty acid in several described bacteria closely related to those from the White Clay Creek sediment clone libraries. These include: *Lysobacter antibioticus* (*Gammaproteobacteria*, aerobic, gram-negative), *Bacillus niacina*, *B. silvestris* (*Firmicutes*, aerobic, gram-positive), *Acidobacterium capsulatum* (*Acidobacteria*, facultative anaerobic, gram-negative), *Flavobacterium aquatile*, *Dysgonomonas gadei*, *Runella slithyformis* (*Bacteroidetes*, aerobic, gram-negative), and *Gemmatimonas aurantiaca* (*Gemmatimonadetes*, aerobic, gram-negative). The fatty acid a15:0 is a major fatty acid in two bacteria closely related to those from the White Clay Creek sediment clone libraries - *Bacillus niacina* and *B. silvestris* (*Firmicutes*, aerobic, gram-positive). The fatty acid 16:0 is widely distributed and found in many of the described bacteria that are closely related to those from the White Clay Creek sediment clone libraries. The fatty acids 10me16:0 is not a major fatty acid of any described bacteria that are closely related to those identified from the White Clay Creek sediment clone libraries. The fatty acid 16:1w9 is a major fatty acid in one bacterium (*Nitrospira cf. moscoviensis* (*Nitrospirae*, aerobic, gram-negative)) that is closely related to those identified from the White Clay Creek sediment clone libraries. The fatty acid 18:1w9 is a major fatty acid in two bacteria (*Acidobacterium capsulatum*, *Acidobacteria*, facultative anaerobic, gram-negative; *Blastopirellula marina*, *Planctomycetes*, facultative anaerobic, gram-
negative) that is closely related to those identified from the White Clay Creek sediment clone libraries. The fatty acid 18:1ω7 is a major fatty acid in several described bacteria closely related to those from the White Clay Creek sediment clone libraries. These include: Burkholderia cepacia, Herbaspirillum rubrisubalbicans, Rhodoferax ferrireducens, Variovorax paradoxus (Betaproteobacteria, aerobic, gram-negative), Nevskia ramosa (Gammaproteobacteria, aerobic, gram-negative) and Filomicrobium fusiforme (Alphaproteobacteria, aerobic, gram-negative). The fatty acid cy19:0 is a major fatty in two described bacteria (Burkholderia cepacia and Filomicrobium fusiforme) closely related to those from the White Clay Creek sediment clone libraries. Assigning microbial identity to the enriched PLFAs using this extended approach, bacteria actively metabolizing stream water DOM were aerobic gram-negative bacteria including species related to Burkholderia cepacia, Nevskia ramosa, Lysobacter antibioticus, Kofleria flava, Filomicrobium fusiforme, Flavobacterium aquatile, Runella slithyformis, Blastoperillula marina Gemmatimonas aurantiacca and Nitrospira cf. moscoviensis; gram-positive or facultative anaerobic gram-negative bacteria including species related to Bacillus niacini and B. silvestris, Rhodoferax ferrireducens, Variovorax paradoxus, Herbaspirillum rubrisubalbicans, Dysgonomonas gadei, and Acidobacterium capsulatum; and anaerobic gram-negative bacteria.

Evaluation of mesocosm approach

Total sediment microbial biomass was increased by removal from White Clay Creek and incubation within the mesocosms; however, this increase was not significant (Figure 1a). Sediment microbial community structure was unchanged by incubation within the mesocosms as neither the percentage that prokaryotes comprised of the total community nor the PC1 score from a PCA analysis of PLFA profiles were significantly different for stream and mesocosm control
sediments. The addition of $^{13}$C-DOM to the natural stream water DOM did not affect either total microbial biomass or microbial community structure. Particularly, the concentration of phospholipid phosphate, the percentage that prokaryotes comprised of the total community and the PC1 score from a PCA analysis of PLFA profiles were not significantly different for mesocosm control and treatment sediments (Figure 1).

**DISCUSSION**

Current studies of DOM metabolism within freshwater streams acknowledge that the structure of sediment microbial communities may modulate the degradation and use of this important carbon and energy resource (e.g. Mineau et al. 2013). However, few, if any, studies directly examine the bacteria that are responsible for the utilization of DOM in streams. In this study, the $^{13}$C incorporated into the microbial phospholipid fatty acids revealed that many, but not all, heterotrophic microorganisms present in White Clay Creek sediments assimilated components from an allochthonous detrital source. The tracer used was designed to mimic, to a far greater extent than any previously used DOC tracer, stream water DOM (Wiegner et al. 2005a). It contains humic and polysaccharide components (Wiegner et al. 2005a), both labile and semi-labile fractions (Wiegner et al. 2005b) and has been used for direct measurement of stream DOC uptake rates coefficients (Kaplan et al. 2008). Kaplan et al. (2008) determined that uptake rate coefficients for the labile and semi-labile fractions were 4.20 and 0.22 km$^{-1}$, respectively, and calculated labile tracer uptake was 272 mg C m$^{-2}$ d$^{-1}$ and semi-labile tracer uptake was 40 mg C m$^{-2}$ d$^{-1}$. These rates were sufficient that all labile tracer DOC and ~25% of semi-labile fraction were taken up during our mesocosm incubations. PLFA-SIP revealed that aerobic gram-negative
bacteria, gram-positive and/or facultative anaerobic gram-negative bacteria, and anaerobic gram-negative bacteria utilized stream water DOM.

Among the microbial PLFAs that showed significant $^{13}$C enrichment, there were two trends - those that were enriched, on average, by 3.5‰ to 5.9‰ and those that were enriched, on average, by 0.8‰ to 1.1‰. The fatty acids showing the strongest enrichment were i15:0, a15:0, 16:1ω9, 16:0, 18:1ω7c and 18:1ω9. Coupling these PLFA-SIP findings with the results of previously constructed 16S rRNA gene sequence clone libraries via published phenotypic species descriptions indicated that several organisms closely related to several described species actively utilized $^{13}$C-labeled leaf leachate (Table 3). Virtually all of these species are known for possessing versatile metabolisms. For example, the *Burkholderia cepacia* complex (Vandamme et al. 1997) is well known for its extraordinary degradative abilities, possessing broad substrate mono- and dioxygenases (Lessie et al. 1996). *Herbaspirillum rubrisubalbicans*, best known as a nitrogen-fixing, plant-growth-promoting rhizobacteria, is also a plant pathogen capable of penetrating plant cell walls (Monteiro et al. 2012). This genus contains a number of aquatic species (e.g. *H. aquaticum*) that can metabolize a wide variety of sugars and other low molecular weight compounds (Dobritsa et al. 2010). *Nevskia ramosa* is considered a neuston bacterium although related OTUs have been identified among the active bacteria present in drinking water biofilms (Keinanen-Toivola et al. 2006). *Nevskia ramosa* is capable of digesting complex organic polymers including starch and cellulose, as well as, many low molecular weight compounds (Sturmeyer et al. 1998). Species of genus *Lysobacter* are typically found in soil and water habitats and *L. antibioticus* is capable of degrading a wide variety of complex substrates including carboxymethyl cellulose, chitin, gelatin, laminarin, protein, Tween-20, Tween-80 and yeast cell walls (Sullivan et al. 2003). $^{13}$C DNA-SIP has shown utilization of 2,4,6-trinitrotoluene by an
OTU related to *L. taiwanensis* in Norfolk Harbor sediments (Gallagher et al. 2010).

*Acidobacteria* are one of the most common bacterial phyla in soil and can also be among the dominant taxa aquatic sediments (Rawat et al. 2012, Spring et al. 2000). *Acidobacterium capsulatum* is known to degrade cellobiose, starch and xylan, and contains homologs to enzymes required for pectin degradation (Rawat et al. 2012). *Bacillus niacini* and *B. silvestris* are known to utilize a host of simple organic molecules, as well as degrade several complex organic molecules. *Flavobacterium aquatile* digests casein and aesculin, and exhibits cystine arylamidase esterase, esterase lipase, and α-glucosidase activity. *Runella slithyformis* is capable of growth on glycogen, D-arabitol, dulcitol, inositol, mannitol, sorbitol, ribose and sorbose and can hydrolyze starch (Copeland et al. 2012). *Blastopirellula marina* digests DNA, aesculin, gelatin and starch, exhibits lipase activity and growth on fructose, glycerol, glutamic acid and chondroitin sulfate (Schlesner et al. 2004). *Gemmatimonas aurantiaca* is capable of growth on yeast extract, polypeptone, succinate, acetate, gelatin, benzoate, glucose, sucrose, galactose, melibiose, maltose, formate and b-hydroxybutyrate (Zhang et al. 2003). Genomic sequencing of *Rhodoferax ferrireducens* indicates that this species possesses highly diverse metabolic capacities including utilization of sugars, acetate and aromatic compounds under both aerobic and anaerobic conditions (Risso et al. 2009). *Variovorax paradoxus* is capable of digesting a wide range of complex organic compounds including (but not limited to) amino acids, polychlorinated biphenyls, dimethylterephthalate, linuron, 2,4-dinitrotoluene, homovanillate, veratraldehyde, 2,4-dichlorophenoxyacetic acid, anthracene, poly(3-hydroxybutyrate), chitin, cellulose, and humic acids. *Dysgonomonas gadei* is known to utilize a wide range of sugars, to hydrolyze starch and aesculin, and to exhibit a wide range of derivative enzyme activities including N-acetyl-b-glucosaminidase, acid phosphatase and trypsin (Hofstad et al. 2000). Combined, the $^{13}$C-
enrichment of many of the abundant fatty acids present in these species, the recovery of gene sequences closely related to these cultured species from clone libraries developed from White Clay Creek and their utilization of labile organic compounds strongly suggests that bacteria related to the species discussed above utilized the labile tracer DOC. The capacity of several species, notably *Rhodoferax ferrireducens, Varivorax paradoxus, Lysobacter antibioticus, Burkholderia cepacia* and *Nevskia ramosa* to digest complex organic compounds suggests that bacteria related to these species are responsible for the utilization of the semi-labile tracer DOC. Among the cultured relatives most closely related to recovered sequences from the sediment community only *Nitrospina moscoviensis* exhibits 16:1ω9 as a dominant fatty acid. *Nitrospina*-like bacteria are nitrite-oxidizing bacteria and members of the deep-branching bacterial phylum *Nitrospirae* with only one class Nitrospira (Bock and Wagner 2006). The enrichment of 16:1ω9 following sediment incubation with 13C-labeled leaf leachate suggests that either species containing 16:1ω9 but not identified from the clone libraries also utilized components of the 13C-labeled leaf leachate or that bacteria closely related to *Nitrospina moscoviensis* exhibit mixotrophic, rather than lithoautotrophic growth in White Clay Creek.

The PLFAs 10me16:0 and cy19:0 showed moderate 13C enrichment following incubation with 13C-labeled leaf leachate. The fatty acid 10me16:0 is viewed as a marker fatty acid for members of the genus *Desulfobacter* within the delta subclass of proteobacteria (Findlay 2004). Macalady et al. (2000) analyzed fatty acid profiles for 100 strains of bacteria including 12 genera of sulfate-reducing bacteria and several other anaerobic species. They concluded that *Desulfobacter* was the major sources of 10me16:0 in environmental samples. The Macalady et al. (2000) analysis also indicated that the PLFA cy19:0 was also strongly associated with *Desulfobacter*. This suggests that sulfate-reducing bacteria, and in particular members of the
genus Desulfo bacter, utilized some component of $^{13}$C-labeled leaf leachate, albeit at a lesser extent compared to those bacteria represented by the strongly labeled PLFAs.

On one occasion, we incubated sediments in the dark and during this trial the $\delta^{13}$C of the PLFAs 20:4$ω$6 and 20:5$ω$3 increased by 3.45‰ and 4.15‰ respectively, compared to an average increase of 1‰ when sediments were incubated under the natural photoperiod. It is important to note that we conducted but a single dark incubation and that 20:4$ω$6 and 20:5$ω$3 are found in both heterotrophic and autotrophic protists. Nevertheless, the presence of label in fatty acids 20:4$ω$6 and 20:5$ω$3 in the dark suggests that protozoan grazers used bacteria that are utilizing $^{13}$C-DOM as food source and may contribute significantly to the transfer of allochthonous carbon via the microbial loop in stream ecosystems. As the system contains both heterotrophic and autotrophic protists, synthesis of 20:4$ω$6 and 20:5$ω$3 by algae during incubations under the natural photoperiod would produce a second, non-labeled source of these fatty acids, which would serve to isotopically dilute those produced by trophic interactions within the sediment microbial community.

Total sediment microbial biomass measured as phospholipid phosphate (nmol PLP gdw$^{-1}$) for the control and $^{13}$C-labeled bioreactors (Table 3) fall within the range (2-280nmol PLP g$^{-1}$ dry wt) of PLP concentrations quantified in freshwater sediments of eastern deciduous forest and those previously published for White Clay Creek (Bott and Kaplan 1985; Sutton and Findlay 2003; Findlay et al. 2008). The increase in microbial biomass in the mesocosm sediments (Figure 1a; comparison T0-TM), though not significant, is likely a response of stream microbial communities to placement within a mesocosm setting. While infrequently assessed and even less frequently discussed (but see Mortazavi et al. 2013, Suárez-Suárez et al. 2011), a ‘mesocosm effect’ appears to be an increase in sediment bacterial abundance. Sediments, in general and streams sediments in
particular, are dynamic and sediment microbial communities experience frequent disturbances associated with storm flows, hydraulic turbulence, or macrofauna activities (Fisher et al., 1982; Schwendel et al., 2011) and are best viewed as rarely, if ever, at maximum biomass. The natural response of sediment microbial communities to disturbance is regrowth leading to increased biomass (Findlay et al., 1990; Traunspurger et al., 1997; Langworthy et al., 2002). In this study, sediments were obtained with the utmost care; however, obtaining a “disturbance free” microbial sediment sample is very difficult, if not impossible. In addition, once placed in the mesocosms sediments were protected from further in-stream disturbances, which, combined, with any disturbance during removal from the stream, likely led to the small observed increase in biomass (Riemann et al. 2000). Stream and mesocosm sediments (T0, TM, and T\textsuperscript{13}C) showed little change in ratios of prokaryotic to eukaryotic biomass. On average, in the bioreactors, ~60 % of the microbial biomass was prokaryotic and the remaining 40 % was eukaryotic, which is well within the range of estimates reported for stream sediments from several low-order forested streams (Sutton and Findlay 2003; Mosher and Findlay 2011) and is similar to previously reported ratios for WCC (Findlay et al. 2008). Thus, there was no change in community structure, or in microbial biomass in these mesocosms in response to sampling procedure or mesocosm effect.

In this study, the additions of 8.73 to 17.47 µg/L of \textsuperscript{13}C-DOM did not significantly alter the structure of the sediment microbial community during any of the mesocosm incubations. In a study where 50 µg C g\textsuperscript{-1} soil of universally \textsuperscript{13}C labeled glucose, glutamine, oxalate or phenol were added to samples of soil, no detectable changes in the soil PLFA profiles were found (Brant et al. 2006). Also, Griffiths et al. (1999) detected no changes in the soil PLFA profiles until rates of additions of a model root exudate exceeded 375µg C g\textsuperscript{-1}d\textsuperscript{-1} in a 14-d experiment. In other studies, the additions of 400 µg C g\textsuperscript{-1} soil of vanillin (Waldrop and Firestone 2004) and 726 µg C g\textsuperscript{-1}
oxalate and glutamate to grassland sandy loam soil (Falchini et al. 2003) resulted in changes in microbial community composition or PLFA profile. The trend in these studies was that as substrate loading increased, relative abundance of specific PLFAs increased leading to changes in total microbial community composition. Our tracer-level substrate addition was significantly smaller than additions used in studies where significant changes in biomass or community composition were observed; this was intentional and designed to avoid changes in biomass and PLFA profiles that could compromise our use of mesocosm-based experimental design.

Though mesocosm experiments have increased our understanding of community ecology, ecosystem dynamics and provided insight into global processes (Fraser and Keddy 1997; Jessup et al. 2004; Cardinale et al. 2006; Benton et al. 2007; Duffy 2009), mesocosms have been criticized as being unrealistic simplifications of natural systems with restricted utility (Carpenter 1996; Schindler 1998; Haag and Matschonat 2001). However, with appropriate scaling, accurate conclusions can be made (Spivak et al. 2011). Our analyses of microbial biomass and community structure indicate that our mesocosm-based experimental design, particularly the comparison of the TM mesocosm control to the T\(^{13}\)C mesocosm treatment samples, was sufficiently robust to warrant examination of individual fatty acids for the incorporation of \(^{13}\)C with the goal of determining the role of sediment microbes in processing streamwater DOM. Also, the significant \(^{13}\)C-enrichment detected in microbial lipids in the T\(^{13}\)C bioreactors clearly demonstrated the high sensitivity of stable isotope probing of PLFA as a technique to elucidate which microbial communities are responsible for the utilization of terrestrial DOC.

In conclusion, the present study provides direct experimental evidence that terrestrial DOM is readily utilized by a broad range of benthic heterotrophic aerobic, facultative anaerobic and anaerobic bacteria in forested headwater streams. We posit that terrestrially derived DOM
exported from forested watersheds is not entirely lost to downstream systems but rather is assimilated and mineralized by a variety of heterotrophic bacteria which, in turn, are grazed by heterotrophic eukaryotes transferring allochthonous carbon and energy to higher trophic levels through the microbial loop (Meyer 1994). Thus, our data have important implications for protection of forested headwater streams where much of the DOM in transport is derived from the surrounding terrestrial ecosystem. As terrestrial DOM is an important source of carbon and energy for stream microbial communities, any human activity that disrupts or accelerates the delivery of terrestrial DOM to headwater streams may need regulation, since perturbation to ecological linkages between aquatic and terrestrial systems could have pronounced effects on microbial community structure and function. This is particularly true where the terrestrial and aquatic ecosystems are tightly linked by large internal fluxes of DOM in the forested landscape (McDowell and Likens 1988; Aitkenhead-Peterson et al. 2003).

Acknowledgements

We thank Janna Brown (University of Alabama, Tuscaloosa, AL) and Sherman Roberts (Stroud Water Research Center, Avondale, PA) for laboratory assistance. Funding for this project was provided by the National Science Foundation (award number DEB-0516235).
REFERENCES


121


Figure 1. Changes in a) microbial biomass, b) percent prokaryotes and c) community structure summarized by PCA axis 1, among treatments and sampling dates for all experiments.

Values are mean differences ± SD, \( n = 6 \). T0-TM= Differences attributed to mesocosm effect, TM-T\(^{13}\)C= Differences attributed to the effects of \(^{13}\)C-labeled DOM.
Table 1. Experimental design of $^{13}$C-DOM uptake experiments.

<table>
<thead>
<tr>
<th>Experiment/ Mesocosm condition</th>
<th>Stream Order/ Sampling Date</th>
<th>$^{13}$C DOM injected (µg/L)</th>
<th>Experiment duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dark</td>
<td>3rd order WCC / 12-15 Oct., 2009</td>
<td>17.47</td>
<td>48</td>
</tr>
<tr>
<td>2. Natural photoperiod</td>
<td>3rd order WCC / 20-23 Oct., 2009</td>
<td>17.47</td>
<td>50</td>
</tr>
<tr>
<td>3. Natural photoperiod</td>
<td>2nd order WCCE/ 3-6 Nov., 2009</td>
<td>17.47</td>
<td>50</td>
</tr>
<tr>
<td>4. Natural photoperiod</td>
<td>2nd order WCCW/ 9-12 Nov., 2009</td>
<td>8.73</td>
<td>50</td>
</tr>
<tr>
<td>5. Natural photoperiod</td>
<td>3rd order WCC/ 20-23 April 2010</td>
<td>8.73</td>
<td>50</td>
</tr>
<tr>
<td>6. Natural photoperiod</td>
<td>3rd order WCC/ 9-12 August 2010</td>
<td>17.47</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 2. Microbial PLFA $\delta^{13}$C values ($\%\epsilon$; mean ± SD) from 6 mesocosm experiments determined using DB-1 and DB-23 chromatographic columns.

<table>
<thead>
<tr>
<th>FAMEs</th>
<th>T0</th>
<th>TM</th>
<th>T$^{13}$C</th>
<th>Mean of d$^a$</th>
<th>n$^b$</th>
<th>significance$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>i15:0</td>
<td>-29.01 ± 0.75</td>
<td>-28.69 ± 0.84</td>
<td>-24.69 ± 3.30</td>
<td>4.00 ± 3.01</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>a15:0</td>
<td>-29.34 ± 0.99</td>
<td>-29.12 ± 0.86</td>
<td>-25.58 ± 3.08</td>
<td>3.54 ± 2.82</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>16:0</td>
<td>-32.35 ± 2.12</td>
<td>-31.89 ± 1.51</td>
<td>-28.12 ± 3.16</td>
<td>3.77 ± 2.92</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>summed feature 1*</td>
<td>-33.16 ± 0.86</td>
<td>-32.50 ± 1.23</td>
<td>-30.20 ± 4.23</td>
<td>2.30 ± 4.57</td>
<td>6</td>
<td>0.23</td>
</tr>
<tr>
<td>16:0, 16:1w9</td>
<td>-33.17 ± 1.81</td>
<td>-32.84 ± 1.49</td>
<td>-28.73 ± 2.81</td>
<td>4.11 ± 2.84</td>
<td>6</td>
<td>0.01</td>
</tr>
<tr>
<td>10me16:0</td>
<td>-30.60 ± 1.76</td>
<td>-29.88 ± 0.49</td>
<td>-28.80 ± 0.72</td>
<td>1.13 ± 0.64</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>cy17:0</td>
<td>-28.53 ± 2.18</td>
<td>-28.70 ± 2.44</td>
<td>-27.84 ± 2.40</td>
<td>0.86 ± 2.10</td>
<td>6</td>
<td>0.55</td>
</tr>
<tr>
<td>summed feature 2*</td>
<td>-31.54 ± 1.52</td>
<td>-31.60 ± 1.40</td>
<td>-27.36 ± 3.36</td>
<td>4.24 ± 2.88</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>18:2w6</td>
<td>-33.07 ± 2.11</td>
<td>-32.83 ± 1.32</td>
<td>-32.09 ± 2.00</td>
<td>0.24 ± 1.85</td>
<td>5</td>
<td>0.50</td>
</tr>
<tr>
<td>18:1w9c, 18:1w7c</td>
<td>-30.35 ± 0.91</td>
<td>-30.56 ± 0.97</td>
<td>-24.70 ± 3.16</td>
<td>5.87 ± 3.03</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>cy19:0</td>
<td>-30.46 ± 0.80</td>
<td>-30.84 ± 0.23</td>
<td>-30.03 ± 0.50</td>
<td>0.82 ± 0.54</td>
<td>6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>20:4w6, 20:5w3</td>
<td>-36.69 ± 1.52</td>
<td>-35.92 ± 1.95</td>
<td>-34.59 ± 1.62</td>
<td>1.33 ± 2.62</td>
<td>6</td>
<td>0.23</td>
</tr>
<tr>
<td>20:4w6, coelluter</td>
<td>-35.25 ± 1.71</td>
<td>-33.85 ± 1.75</td>
<td>-32.42 ± 4.29</td>
<td>0.40 ± 4.73</td>
<td>5</td>
<td>0.50</td>
</tr>
<tr>
<td>20:5w3, coelluter</td>
<td>-37.26 ± 1.44</td>
<td>-35.92 ± 2.01</td>
<td>-34.75 ± 1.38</td>
<td>1.17 ± 2.81</td>
<td>6</td>
<td>0.27</td>
</tr>
<tr>
<td>22:6w3</td>
<td>-33.85 ± 1.49</td>
<td>-33.40 ± 1.62</td>
<td>-33.29 ± 1.68</td>
<td>0.05 ± 2.85</td>
<td>4</td>
<td>0.92</td>
</tr>
</tbody>
</table>

T0 = natural sediment control, TM= experimental control, T$^{13}$C= treatment sediment. * Summed feature 1 includes 16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t; summed feature 2 includes 18:2ω6, 18:3w3, 18:1ω9, 18:1ω7c, 18:1ω5, $^a$=difference in $\delta^{13}$C values of T$^{13}$C- TM, $^b$= number of time when peak was present in all three treatments per experiment, $^c$=p of paired t-test.
Table 3. Phylogenetic affiliation of bacterial fatty acids functional groups extracted from White Clay Creek sediment*

<table>
<thead>
<tr>
<th>Phylum or Subphylum</th>
<th>Species</th>
<th>Fatty acids</th>
<th>Morphology</th>
<th>Metabolism</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaproteobacteria</td>
<td><em>Rhodoferax ferrireducens</em></td>
<td>16:0, 16:1w7, 18:1w7</td>
<td>Gram -ve</td>
<td>aerobic, facultative anaerobic</td>
<td>Hahn et al., 2010</td>
</tr>
<tr>
<td></td>
<td><em>Variovorax paradoxus</em></td>
<td>16:0, 16:1w7, cy17:0, 18:1w7</td>
<td>Gram -ve</td>
<td>aerobic, facultative anaerobic</td>
<td>Jin et al., 2012</td>
</tr>
<tr>
<td></td>
<td><em>Herbaspirillum rubrisubalbicans</em></td>
<td>16:1w7, 16:0, 18:1w7</td>
<td>Gram -ve</td>
<td>aerobic, facultative anaerobic</td>
<td>Jung et al. 2007</td>
</tr>
<tr>
<td></td>
<td><em>Burkholderia cepacia</em></td>
<td>16:0, 16:1w7, cy19:0</td>
<td>Gram -ve</td>
<td>strict aerobe</td>
<td>Stead, 1992</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td><em>Lysobacter antibioticus</em></td>
<td>i15:0, i17:1w9</td>
<td>Gram -ve</td>
<td>aerobic</td>
<td>Srinivasan et al., 2010</td>
</tr>
<tr>
<td></td>
<td><em>Nevskia ramosa</em></td>
<td>16:1w7, 16:0, 18:1w7</td>
<td>Gram -ve</td>
<td>aerobic</td>
<td>Losey et al., 2013</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>“Polyangium vitellum” [Kofleria flavo] [Haliangium ochraceum]</td>
<td>i16:0, 16:0</td>
<td>Gram -ve</td>
<td>strict aerobe</td>
<td>Fudou et al., 2002</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>“Pedomicrobium fusiforme” [Filomicrobium fusiforme]</td>
<td>18:1w7, 16:0, cy19:0</td>
<td>Gram –ve</td>
<td>aerobe</td>
<td>Wu et al., 2009</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td><em>Flavobacterium aquatile</em></td>
<td>i15:0, a15:0, 15:1w6</td>
<td>Gram -ve</td>
<td>strict aerobic</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td></td>
<td><em>Dysgonomonas gadei</em></td>
<td>i14:0, i15:0, 16:0</td>
<td>Gram -ve</td>
<td>aerobic, facultative anaerobic</td>
<td>Hofstad et al., 2000</td>
</tr>
<tr>
<td></td>
<td><em>Runella slithyformis</em></td>
<td>a15:0, i15:0, 16:1w5</td>
<td>Gram -ve</td>
<td>strict aerobe</td>
<td>Copeland et al., 2012</td>
</tr>
<tr>
<td>Firmicutes</td>
<td><em>Bacillus niacini</em></td>
<td>a15:0, i15:0, 16:0, 16:1w11, 18:0</td>
<td>Gram +ve</td>
<td>facultative anaerobe</td>
<td>Hong et al., 2012</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus silvestris</em></td>
<td>i15:0, i16:1</td>
<td>Gram +ve</td>
<td>aerobic</td>
<td>Reddy et al., 2008</td>
</tr>
<tr>
<td></td>
<td><em>Dendrosporobacter quercicus</em></td>
<td>15:1, 17:1</td>
<td>Gram -ve</td>
<td>anaerobic</td>
<td>Strompl et al., 2000</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td><em>Acidobacterium capsulatum</em></td>
<td>i15:0, 18:1w9</td>
<td>Gram -ve</td>
<td>facultative anaerobic</td>
<td>Kulichevskaya et al., 2012</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td><em>Pirellula (Blastopirellula) marina</em></td>
<td>16:0, 18:1w9</td>
<td>Gram -ve</td>
<td>strict aerobe</td>
<td>Schlesner et al., 2004</td>
</tr>
<tr>
<td>Nitrospirae</td>
<td><em>Nitrospina moscoviensis</em></td>
<td>16:1w9, 16:0</td>
<td>Gram -ve</td>
<td>aerobe</td>
<td>Lipski et al., 2001, Spieck et al., 2006</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td><em>Gemmatimonas aurantiaca</em></td>
<td>i15:0, 16:1, 14:0</td>
<td>Gram -ve</td>
<td>aerobe</td>
<td>Zhang et al., 2003</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td><em>Kutzneria kofuensis</em></td>
<td>i16:0 (10Me17:0)</td>
<td>Gram +ve</td>
<td>aerobe</td>
<td>Stackebrandt et al., 1994, Suriyachadkun et al., 2013</td>
</tr>
</tbody>
</table>

*fatty acids that showed $^{13}$C enrichment in this study are in bold type
# Hullar et al., (2006) bacterial 16S rRNA gene clone libraries
CHAPTER 5

OVERALL CONCLUSIONS

Microbial communities are important players in lotic ecosystems and provide a critical link to higher trophic levels through the microbial loop (Pomeroy 1974; Hall and Meyer 1998). They are responsible for several biogeochemical transformations, including DOC uptake, degradation, and mineralization (Kaplan and Newbold 1993; Pusch et al. 1998; Fischer and Pusch 2001; Tank et al. 2010). Thus, DOC is a major energy source for benthic microbial metabolism and drives lotic ecosystem processes and maintains secondary production of consumers. To better understand the ecology of microbial communities, it is important not only to describe the community composition and identify environmental factors that regulate their spatial and temporal variations, but also to elucidate which of these microbes within the community actively participate in DOC uptake, degradation, and mineralization. Studies that have investigated bacterial utilization of DOC have indicated its heterogeneous nature being comprised of both labile components, which turn over rapidly, and refractory components, which turn over more slowly (Moran and Hodson 1990). The refractory DOC pool is composed of larger molecules, primarily humic in nature, and often assumed to be largely inert to bacterial degradation. However, few studies have shown that a portion of the humic substances is biologically degradable (Moran and Hodson 1990; Carlsson et al. 1999).
The goals of this interdisciplinary study were to elucidate the bacteria responsible for utilization of humic DOC in streams and to assess overall variability in microbial biomass, carbon isotope signatures and community structure over time and across multiple spatial scales in stream networks. Moreover, I examined the role of environmental heterogeneity and hydrological indices in structuring benthic microbial communities. This dissertation was arranged into five chapters with chapters one and five (this chapter) providing a comprehensive introduction and summary, respectively.

Chapter two examined spatial variations in sedimentary microbial biomass and community structure of forested streams within two distinct watersheds. A nested sampling design was used to sample sediments from 1st- through 3rd - order streams in White Clay Creek watershed and from 1st, 3rd and 5th- order streams in the Neversink watershed across multiple spatial scales. PLFA analysis was used to characterize the microbial biomass and communities found in the samples from these streams. In general, streams from the White Clay Creek watershed showed higher total microbial biomass, percent prokaryotes and bacterial abundance than those within the Neversink watershed. Also, C:N ratios and conductivity were generally higher in the White Clay Creek watershed compared to the Neversink watershed. In addition, C:N ratios were higher in 1st and 2nd order streams and lower in 3rd and 5th order stream sediments. The variation in microbial biomass in stream sediments correlated with C:N ratio, sediment grain size, percent carbon and percent water content. In contrast to reach-scale similarity in microbial biomass, there were large significant differences in biomass at stream-scale and between watersheds. Sediment microbial community structure in the fourteen streams investigated displayed distinct watershed-scale variations at the scale of hundreds of kilometers and among-stream within a watershed variation at the scale of hundreds of meter. Also, the shift from
predominance of bacteria in lower order streams to phototrophic microeukaryotes in higher order streams may be explained by decreased importance of terrestrial organic inputs from riparian vegetation and increased importance of algal production downstream. Overall, the magnitude of within stream variation in microbial biomass was small compared to the variability noted among streams and between watersheds. Furthermore, this study and others (Oda et al. 2003; Crump et al. 2004; Hughes-Martiny et al. 2006; Fierer et al. 2007) conducted over a range of spatial scales implies non-random distribution of microbial community and that environmental heterogeneity and geographical distance can structure microbial biomass and distribution.

Chapter three provided baseline information on the natural abundance and seasonal variation in compound specific stable carbon isotope signatures of individual microbial PLFAs of White Clay Creek. Also, seasonal variation of microbial biomass and community structure was investigated with assessment of the effect of hydrological and environmental variables on microbial communities in headwater stream sediments. This work demonstrated that sedimentary microbial biomass was seasonally dynamic and significantly correlated to a combination of high and low flood pulse counts, variability in daily flow and DOC concentrations. The seasonal pattern of variation observed in microbial community structure was as a result of a shift between the ratios of prokaryotic to eukaryotic component of the community. This shift was significantly correlated with seasonal changes in median daily flow, high and low flood pulse counts, DOC concentrations and water temperature. Stable carbon isotopes signatures of some PLFAs varied significantly over an annual cycle. Both bacterial and microeukaryotic stable carbon isotope signatures were heaviest in the spring and lightest in autumn or winter. Some bacterial lipids were isotopically depleted on average by 2-5‰ relative to δ¹³C of total organic carbon and enriched relative to algae PLFAs. During spring bloom, increased algal productivity partly due to moderate
stream temperature and flow, and reduced input of CO2 from detrital decomposition appears to result in a more negative δ13C PLFAs (Finlay 2004). Thus, the annual variation of the δ13C of biota is sensitive in providing indication of changes resulting from ecological processes related to ecosystem functions. In addition, this findings support emerging picture in microbial ecology for non-random patterns in microbial biomass and community structure and that local environmental heterogeneity regulate microbial distribution over annual cycles.

Chapter four elucidates which heterotrophic benthic microbes within streams actively utilize humic DOC and ultimately control the material flux that influences higher trophic levels. Streambed sediments were incubated in re-circulating mesocosm chambers amended with leachate from composted 13C-labelled tulip poplar tree-tissues (a process that yields 13C-labeled DOM with size and lability fractions approximating streamwater DOM) and examined for 13C incorporation into microbial PLFA. Total community structure and metabolically active community members from the mesocosm incubated sediments were elucidated through PLFA and 13C isotopic analysis of the microbial PLFAs respectively. This work demonstrated that the mesocosm-based experimental design, particularly the comparison of the TM mesocosm control to the T13C mesocosm treatment samples, is sufficiently robust to warrant examination of individual fatty acids for the incorporation of 13C labeled DOC into microbial lipids. Bacterial fatty acids i15:0, a15:0, 16:0, 16:1ω9, 18:1ω9c, 18:1ω7c, 10me16 and cy19:0 (aerobic, anaerobic and facultative anaerobic bacteria biomarkers) and fatty acids 20:4ω6, 20:5ω3 (microeukaryotic biomarkers) showed increased abundance of 13C. The fatty acids showing the strongest enrichment were i15:0, a15:0, 16:1ω9, 16:0, 18:1ω7c and 18:1ω9. These are marker fatty acids for members of major bacterial groups such as Alphaproteobacteria, Gammaproteobacteria, Firmacutes, Acidobacteria, Bacteroidetes, Gemmatimonadetes and Nitrospirae. These species are known for
versatile metabolisms and PLFA-SIP findings suggest that they actively utilized $^{13}$C-labeled leaf leachate. The PLFAs 10me16:0 and cy19:0 showed moderate $^{13}$C enrichment and are marker fatty acids for *Desulfobacter*. This suggests that sulfate-reducing bacteria, in particular, members of the genus *Desulfobacter* utilized some component of $^{13}$C-labeled leaf leachate. In addition, the presence of label in fatty acids 20:4ω6 and 20:5ω3 suggests that protozoan grazers used bacteria that are utilizing $^{13}$C-DOM as food source and may contribute significantly to the transfer of allochthonous carbon via microbial loop in stream ecosystems. This work suggests that these benthic microbes are important players within steam ecosystems with regards to humic DOC uptake, degradation, respiration, and transfer to higher trophic levels.

The overall results of this study shows evidence that the sedimentary microbial community displayed seasonal pattern of variation in structure and isotopic signatures, distinct watershed-level biogeography, as well as variation along a headwater streams-large stream gradient. The variation observed in sedimentary microbial community structure in time and space was significantly correlated to local environmental heterogeneity. Further, this study provides direct experimental evidence that benthic microbial communities in forested headwater streams readily utilize humic DOM. In summary, this study depicts a complex relationship between microbial community structure, environmental heterogeneity and utilization of humic DOM, indicating that terrestrial DOM quality and quantity along with other hydro-ecological variables should be considered among the important factors that structure benthic microbial communities in lotic ecosystems.

For future directions, investigation should be focused on examining spatial patterns in sedimentary microbial distribution at larger spatial scales, such as within and among watersheds in a biome and in different biomes with samplings at different times of the year. Results from
such extensive studies ranging from small scales (<10km) to intermediate (10 – 3000km) and at larger spatial scales (>10,000km) would provide addition information about the role of environmental heterogeneity and geographical distance in structuring microbial community structure. Also, such study can be complemented with research efforts to identify which microbial individual populations are unique to each habitat using molecular techniques. Results from such undertakings can provide useful insights into the long held assumption in microbiology of “everything is everywhere, but the environment selects” (Bass-Becking, 1934).

Further investigations on the mesocosm experiment should be focused on scaling up to whole-stream tracer- level isotope addition with sufficiently enriched $^{13}$C-DOC to examine the incorporation of labeled $^{13}$C-DOC into stream sediment microbial lipids. Undoubtedly, this will be a challenging task but such study would provide a strong support for the elucidation of the metabolically active microbial communities within stream sediments involved in humic DOC metabolism as shown in this study. Furthermore, results from such study will advance our understanding of methods for measuring in situ microbial and stream dynamics.
REFERENCES


Supplementary Data

Appendix 1. Values of the 108 hydrologic indices used in the study

<table>
<thead>
<tr>
<th>Code</th>
<th>Hydrologic Index</th>
<th>Unit</th>
<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
<th>Year 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDF</td>
<td>Mean daily flows</td>
<td>m³s⁻¹</td>
<td>0.37</td>
<td>0.35</td>
<td>0.56</td>
<td>0.58</td>
<td>0.43</td>
</tr>
<tr>
<td>MQ50</td>
<td>Median daily flow</td>
<td>m³s⁻¹</td>
<td>0.31</td>
<td>0.28</td>
<td>0.42</td>
<td>0.31</td>
<td>0.38</td>
</tr>
<tr>
<td>MVD1</td>
<td>Variability in daily flow 1</td>
<td>(−)</td>
<td>1.16</td>
<td>1.12</td>
<td>1.35</td>
<td>2.28</td>
<td>1.02</td>
</tr>
<tr>
<td>MVD2</td>
<td>Variability in daily flow 2</td>
<td>(−)</td>
<td>-0.38</td>
<td>-0.28</td>
<td>-0.63</td>
<td>-0.49</td>
<td>-0.48</td>
</tr>
<tr>
<td>MDSK</td>
<td>Skewness in daily flows</td>
<td>(−)</td>
<td>1.19</td>
<td>1.27</td>
<td>1.32</td>
<td>1.86</td>
<td>1.12</td>
</tr>
<tr>
<td>MR1</td>
<td>Ranges in daily flows 1</td>
<td>(−)</td>
<td>0.28</td>
<td>0.36</td>
<td>0.17</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>MR2</td>
<td>Ranges in daily flows 2</td>
<td>(−)</td>
<td>0.42</td>
<td>0.58</td>
<td>0.29</td>
<td>0.41</td>
<td>0.31</td>
</tr>
<tr>
<td>MR3</td>
<td>Ranges in daily flows 3</td>
<td>(−)</td>
<td>0.48</td>
<td>0.67</td>
<td>0.35</td>
<td>0.45</td>
<td>0.41</td>
</tr>
<tr>
<td>MS1</td>
<td>Spreads in daily flows 1</td>
<td>(−)</td>
<td>-1.08</td>
<td>-0.81</td>
<td>-2.09</td>
<td>-1.20</td>
<td>-1.49</td>
</tr>
<tr>
<td>MS2</td>
<td>Spreads in daily flows 2</td>
<td>(−)</td>
<td>-0.75</td>
<td>-0.42</td>
<td>-1.44</td>
<td>-0.76</td>
<td>-1.22</td>
</tr>
<tr>
<td>MS3</td>
<td>Spreads in daily flows 3</td>
<td>(−)</td>
<td>-0.63</td>
<td>-0.32</td>
<td>-1.23</td>
<td>-0.69</td>
<td>-0.94</td>
</tr>
<tr>
<td>MM1</td>
<td>Mean monthly flow - October</td>
<td>m³s⁻¹</td>
<td>0.28</td>
<td>0.20</td>
<td>0.63</td>
<td>0.55</td>
<td>0.51</td>
</tr>
<tr>
<td>MM2</td>
<td>Mean monthly flow - November</td>
<td>m³s⁻¹</td>
<td>0.25</td>
<td>0.24</td>
<td>0.34</td>
<td>0.29</td>
<td>0.69</td>
</tr>
<tr>
<td>MM3</td>
<td>Mean monthly flow - December</td>
<td>m³s⁻¹</td>
<td>0.48</td>
<td>0.62</td>
<td>1.14</td>
<td>0.39</td>
<td>0.86</td>
</tr>
<tr>
<td>MM4</td>
<td>Mean monthly flow - January</td>
<td>m³s⁻¹</td>
<td>0.31</td>
<td>0.36</td>
<td>0.69</td>
<td>0.31</td>
<td>0.69</td>
</tr>
<tr>
<td>MM5</td>
<td>Mean monthly flow - February</td>
<td>m³s⁻¹</td>
<td>0.77</td>
<td>0.34</td>
<td>0.61</td>
<td>0.70</td>
<td>0.50</td>
</tr>
<tr>
<td>MM6</td>
<td>Mean monthly flow - March</td>
<td>m³s⁻¹</td>
<td>0.62</td>
<td>0.27</td>
<td>1.22</td>
<td>0.96</td>
<td>0.43</td>
</tr>
<tr>
<td>MM7</td>
<td>Mean monthly flow - April</td>
<td>m³s⁻¹</td>
<td>0.43</td>
<td>0.41</td>
<td>0.72</td>
<td>0.71</td>
<td>0.39</td>
</tr>
<tr>
<td>MM8</td>
<td>Mean monthly flow - May</td>
<td>m³s⁻¹</td>
<td>0.42</td>
<td>0.49</td>
<td>0.46</td>
<td>0.46</td>
<td>0.33</td>
</tr>
<tr>
<td>MM9</td>
<td>Mean monthly flow - June</td>
<td>m³s⁻¹</td>
<td>0.25</td>
<td>0.37</td>
<td>0.27</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>MM10</td>
<td>Mean monthly flow - July</td>
<td>m³s⁻¹</td>
<td>0.24</td>
<td>0.22</td>
<td>0.32</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>MM11</td>
<td>Mean monthly flow - August</td>
<td>m³s⁻¹</td>
<td>0.16</td>
<td>0.36</td>
<td>0.16</td>
<td>0.88</td>
<td>0.15</td>
</tr>
<tr>
<td>MM12</td>
<td>Mean monthly flow - September</td>
<td>m³s⁻¹</td>
<td>0.27</td>
<td>0.37</td>
<td>0.15</td>
<td>1.29</td>
<td>0.17</td>
</tr>
<tr>
<td>MMV1</td>
<td>Variability in monthly flows-October</td>
<td>(--)</td>
<td>1.57</td>
<td>0.43</td>
<td>1.50</td>
<td>2.82</td>
<td>0.23</td>
</tr>
<tr>
<td>MMV2</td>
<td>Variability in monthly flows-November</td>
<td>(--)</td>
<td>0.27</td>
<td>0.51</td>
<td>0.25</td>
<td>0.48</td>
<td>1.25</td>
</tr>
<tr>
<td>MMV3</td>
<td>Variability in monthly flows-December</td>
<td>(--)</td>
<td>0.76</td>
<td>1.55</td>
<td>1.52</td>
<td>0.94</td>
<td>0.87</td>
</tr>
<tr>
<td>MMV4</td>
<td>Variability in monthly flows-January</td>
<td>(--)</td>
<td>0.23</td>
<td>0.71</td>
<td>0.91</td>
<td>0.53</td>
<td>0.75</td>
</tr>
<tr>
<td>MMV5</td>
<td>Variability in monthly flows-February</td>
<td>(--)</td>
<td>1.52</td>
<td>0.35</td>
<td>0.50</td>
<td>0.59</td>
<td>0.28</td>
</tr>
<tr>
<td>MMV6</td>
<td>Variability in monthly flows-March</td>
<td>(--)</td>
<td>0.63</td>
<td>0.09</td>
<td>0.83</td>
<td>1.40</td>
<td>0.28</td>
</tr>
<tr>
<td>MMV7</td>
<td>Variability in monthly flow - April</td>
<td>(--)</td>
<td>0.31</td>
<td>0.52</td>
<td>0.23</td>
<td>0.62</td>
<td>0.66</td>
</tr>
<tr>
<td>MMV8</td>
<td>Variability in monthly flows-May</td>
<td>(--)</td>
<td>0.34</td>
<td>1.09</td>
<td>0.19</td>
<td>0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>MMV9</td>
<td>Variability in monthly flows-June</td>
<td>(--)</td>
<td>0.32</td>
<td>0.64</td>
<td>0.19</td>
<td>0.16</td>
<td>0.46</td>
</tr>
<tr>
<td>MMV10</td>
<td>Variability in monthly flows-July</td>
<td>(--)</td>
<td>0.52</td>
<td>0.23</td>
<td>0.98</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>MMV11</td>
<td>Variability in monthly flows-August</td>
<td>(--)</td>
<td>0.29</td>
<td>0.71</td>
<td>0.19</td>
<td>3.37</td>
<td>0.22</td>
</tr>
<tr>
<td>MMV12</td>
<td>Variability in monthly flows-September</td>
<td>(--)</td>
<td>1.23</td>
<td>1.38</td>
<td>1.25</td>
<td>2.00</td>
<td>0.63</td>
</tr>
<tr>
<td>MV1</td>
<td>Variability across monthly flows1</td>
<td>(--)</td>
<td>4.87</td>
<td>5.33</td>
<td>5.34</td>
<td>12.46</td>
<td>3.74</td>
</tr>
<tr>
<td>MV2</td>
<td>Variability across monthly flows2</td>
<td>(--)</td>
<td>0.29</td>
<td>0.32</td>
<td>0.37</td>
<td>0.38</td>
<td>0.21</td>
</tr>
<tr>
<td>MV3</td>
<td>Variability across monthly flows3</td>
<td>(--)</td>
<td>0.84</td>
<td>1.06</td>
<td>1.11</td>
<td>1.42</td>
<td>0.87</td>
</tr>
<tr>
<td>MV4</td>
<td>Variability across monthly flows4</td>
<td>(--)</td>
<td>75.01</td>
<td>79.30</td>
<td>82.53</td>
<td>145.59</td>
<td>62.08</td>
</tr>
<tr>
<td>MMSK</td>
<td>Skewness in monthly flows</td>
<td>(--)</td>
<td>0.23</td>
<td>0.31</td>
<td>0.35</td>
<td>0.61</td>
<td>0.23</td>
</tr>
<tr>
<td>MAR</td>
<td>Mean annual runoff</td>
<td>m³ s⁻¹ km⁻²</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Low flow condition**

<p>| MML1 | Mean minimum monthly flow-October | m³ s⁻¹ | 0.12 | 0.14 | 0.19 | 0.21 | 0.40 |
| MML2 | Mean minimum monthly flow-November | m³ s⁻¹ | 0.19 | 0.17 | 0.26 | 0.21 | 0.37 |
| MML3 | Mean minimum monthly flow-December | m³ s⁻¹ | 0.24 | 0.22 | 0.34 | 0.24 | 0.51 |
| MML4 | Mean minimum monthly flow-January | m³ s⁻¹ | 0.22 | 0.25 | 0.45 | 0.22 | 0.48 |
| MML5 | Mean minimum monthly flow-February | m³ s⁻¹ | 0.31 | 0.25 | 0.42 | 0.28 | 0.40 |</p>
<table>
<thead>
<tr>
<th>Flow Condition</th>
<th>MML6</th>
<th>Mean Minimum Monthly Flow - March</th>
<th>m³ s⁻¹</th>
<th>0.40</th>
<th>0.23</th>
<th>0.65</th>
<th>0.40</th>
<th>0.34</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MML7</td>
<td>Mean Minimum Monthly Flow - April</td>
<td>m³ s⁻¹</td>
<td>0.31</td>
<td>0.26</td>
<td>0.54</td>
<td>0.45</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>MML8</td>
<td>Mean Minimum Monthly Flow - May</td>
<td>m³ s⁻¹</td>
<td>0.28</td>
<td>0.27</td>
<td>0.34</td>
<td>0.31</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>MML9</td>
<td>Mean Minimum Monthly Flow - June</td>
<td>m³ s⁻¹</td>
<td>0.17</td>
<td>0.23</td>
<td>0.18</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>MML10</td>
<td>Mean Minimum Monthly Flow - July</td>
<td>m³ s⁻¹</td>
<td>0.15</td>
<td>0.17</td>
<td>0.15</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MML11</td>
<td>Mean Minimum Monthly Flow - August</td>
<td>m³ s⁻¹</td>
<td>0.12</td>
<td>0.19</td>
<td>0.10</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>MML12</td>
<td>Mean Minimum Monthly Flow - September</td>
<td>m³ s⁻¹</td>
<td>0.12</td>
<td>0.20</td>
<td>0.08</td>
<td>0.26</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>MBI</td>
<td>Base Flow Index</td>
<td>(--)</td>
<td>0.32</td>
<td>0.41</td>
<td>0.17</td>
<td>0.22</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>MBV</td>
<td>Variability in Base Flow Index</td>
<td>(--)</td>
<td>3.00</td>
<td>2.47</td>
<td>5.42</td>
<td>2.57</td>
<td>9.88</td>
</tr>
<tr>
<td></td>
<td>MAL</td>
<td>Specific Mean Annual Minimum Flows</td>
<td>m³ s⁻¹ km⁻²</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>High Flow Condition</td>
<td>MMH1</td>
<td>Mean Maximum Monthly Flow - October</td>
<td>m³ s⁻¹</td>
<td>2.66</td>
<td>0.54</td>
<td>4.47</td>
<td>8.92</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>MMH2</td>
<td>Mean Maximum Monthly Flow - November</td>
<td>m³ s⁻¹</td>
<td>0.51</td>
<td>0.76</td>
<td>0.68</td>
<td>0.96</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td>MMH3</td>
<td>Mean Maximum Monthly Flow - December</td>
<td>m³ s⁻¹</td>
<td>2.24</td>
<td>5.55</td>
<td>7.87</td>
<td>1.87</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>MMH4</td>
<td>Mean Maximum Monthly Flow - January</td>
<td>m³ s⁻¹</td>
<td>0.57</td>
<td>1.67</td>
<td>3.96</td>
<td>1.05</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>MMH5</td>
<td>Mean Maximum Monthly Flow - February</td>
<td>m³ s⁻¹</td>
<td>6.46</td>
<td>0.74</td>
<td>1.56</td>
<td>2.18</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>MMH6</td>
<td>Mean Maximum Monthly Flow - March</td>
<td>m³ s⁻¹</td>
<td>2.44</td>
<td>0.31</td>
<td>6.03</td>
<td>7.73</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>MMH7</td>
<td>Mean Maximum Monthly Flow - April</td>
<td>m³ s⁻¹</td>
<td>0.93</td>
<td>1.19</td>
<td>1.33</td>
<td>2.44</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>MMH8</td>
<td>Mean Maximum Monthly Flow - May</td>
<td>m³ s⁻¹</td>
<td>0.82</td>
<td>3.23</td>
<td>0.65</td>
<td>0.88</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>MMH9</td>
<td>Mean Maximum Monthly Flow - June</td>
<td>m³ s⁻¹</td>
<td>0.57</td>
<td>1.42</td>
<td>0.37</td>
<td>0.45</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>MMH10</td>
<td>Mean Maximum Monthly Flow - July</td>
<td>m³ s⁻¹</td>
<td>0.85</td>
<td>0.40</td>
<td>1.87</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>MMH11</td>
<td>Mean Maximum Monthly Flow - August</td>
<td>m³ s⁻¹</td>
<td>0.34</td>
<td>1.02</td>
<td>0.27</td>
<td>16.68</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>MMH12</td>
<td>Mean Maximum Monthly Flow - September</td>
<td>m³ s⁻¹</td>
<td>1.93</td>
<td>2.94</td>
<td>1.10</td>
<td>13.56</td>
<td>0.59</td>
</tr>
<tr>
<td>Parameter</td>
<td>Description</td>
<td>Units</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>MHF1</td>
<td>High flow discharge 1</td>
<td></td>
<td>0.39</td>
<td>0.53</td>
<td>0.23</td>
<td>0.41</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>MHF2</td>
<td>High flow discharge 2</td>
<td></td>
<td>0.49</td>
<td>0.65</td>
<td>0.36</td>
<td>0.62</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>MHF3</td>
<td>High flow discharge 3</td>
<td></td>
<td>0.65</td>
<td>0.82</td>
<td>0.51</td>
<td>0.77</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>MHA</td>
<td>Specific mean annual maximum flows</td>
<td>m³ s⁻¹ km⁻²</td>
<td>0.22</td>
<td>0.19</td>
<td>0.27</td>
<td>0.57</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>MHV1</td>
<td>High flow volume 1</td>
<td>days</td>
<td>5.17</td>
<td>7.55</td>
<td>9.61</td>
<td>17.43</td>
<td>7.04</td>
<td></td>
</tr>
<tr>
<td>MHV2</td>
<td>High flow volume 2</td>
<td>days</td>
<td>11.70</td>
<td>10.85</td>
<td>14.24</td>
<td>26.54</td>
<td>13.86</td>
<td></td>
</tr>
<tr>
<td>MHV3</td>
<td>High flow volume 3</td>
<td>days</td>
<td>13.79</td>
<td>28.16</td>
<td>21.46</td>
<td>52.88</td>
<td>20.24</td>
<td></td>
</tr>
<tr>
<td>MHP1</td>
<td>High peak flow 1</td>
<td></td>
<td>1.82</td>
<td>1.99</td>
<td>2.10</td>
<td>3.08</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>MHP2</td>
<td>High peak flow 2</td>
<td></td>
<td>7.25</td>
<td>5.98</td>
<td>6.60</td>
<td>10.05</td>
<td>6.52</td>
<td></td>
</tr>
<tr>
<td>MHP3</td>
<td>High peak flow 3</td>
<td></td>
<td>10.42</td>
<td>14.08</td>
<td>12.03</td>
<td>20.97</td>
<td>9.57</td>
<td></td>
</tr>
<tr>
<td>MHP4</td>
<td>High peak flow 4</td>
<td></td>
<td>2.50</td>
<td>2.61</td>
<td>2.99</td>
<td>5.01</td>
<td>2.32</td>
<td></td>
</tr>
</tbody>
</table>

**Frequency of flow events**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Units</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLC</td>
<td>Low flood pulse count</td>
<td>year⁻¹</td>
<td>78.00</td>
<td>88.00</td>
<td>87.00</td>
<td>77.00</td>
<td>91.00</td>
<td></td>
</tr>
<tr>
<td>FLS</td>
<td>Frequency of low flow spell</td>
<td>year⁻¹</td>
<td>16.00</td>
<td>15.00</td>
<td>19.00</td>
<td>16.00</td>
<td>12.00</td>
<td></td>
</tr>
<tr>
<td>FHC1</td>
<td>High flood pulse count 1</td>
<td>year⁻¹</td>
<td>78.00</td>
<td>78.00</td>
<td>91.00</td>
<td>80.00</td>
<td>76.00</td>
<td></td>
</tr>
<tr>
<td>FHC2</td>
<td>High flood pulse count 2</td>
<td>year⁻¹</td>
<td>10.00</td>
<td>16.00</td>
<td>12.00</td>
<td>29.00</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>FHC3</td>
<td>High flood pulse count 3</td>
<td>year⁻¹</td>
<td>5.00</td>
<td>3.00</td>
<td>7.00</td>
<td>10.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>FRE1</td>
<td>Flood frequency 1</td>
<td>year⁻¹</td>
<td>164.00</td>
<td>136.00</td>
<td>192.00</td>
<td>174.00</td>
<td>183.00</td>
<td></td>
</tr>
<tr>
<td>FRE2</td>
<td>Flood frequency 2</td>
<td>year⁻¹</td>
<td>10.00</td>
<td>16.00</td>
<td>21.00</td>
<td>29.00</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>FRE3</td>
<td>Flood frequency 3</td>
<td>year⁻¹</td>
<td>5.00</td>
<td>3.00</td>
<td>7.00</td>
<td>10.00</td>
<td>4.00</td>
<td></td>
</tr>
<tr>
<td>FRE4</td>
<td>Flood frequency 4</td>
<td>year⁻¹</td>
<td>271.00</td>
<td>261.00</td>
<td>270.00</td>
<td>270.00</td>
<td>273.00</td>
<td></td>
</tr>
<tr>
<td>FRE5</td>
<td>Flood frequency 5</td>
<td>year⁻¹</td>
<td>78.00</td>
<td>102.00</td>
<td>91.00</td>
<td>80.00</td>
<td>76.00</td>
<td></td>
</tr>
</tbody>
</table>

**Duration of flow events**

*Average flow conditions*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Units</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLE1</td>
<td>Low exceedence flows 1</td>
<td></td>
<td>1.36</td>
<td>1.22</td>
<td>1.47</td>
<td>1.73</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>DLE2</td>
<td>Low exceedence flows 2</td>
<td></td>
<td>1.73</td>
<td>1.84</td>
<td>2.13</td>
<td>2.51</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>DL0</td>
<td>Number of zero-flow days</td>
<td>year⁻¹</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>DLP0</td>
<td>Percent of zero-flow months</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

**High flow conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Units</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFD</td>
<td>Flood duration 1</td>
<td></td>
<td>1.83</td>
<td>1.55</td>
<td>2.10</td>
<td>1.90</td>
<td>1.79</td>
<td></td>
</tr>
<tr>
<td>DHP</td>
<td>High flow pulse duration</td>
<td>days</td>
<td>0.78</td>
<td>0.72</td>
<td>1.27</td>
<td>1.56</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>DHPV</td>
<td>Variability in high flow pulse duration</td>
<td></td>
<td>102.98</td>
<td>102.20</td>
<td>98.67</td>
<td>166.34</td>
<td>85.89</td>
<td></td>
</tr>
<tr>
<td>DHF1</td>
<td>High flow duration 1</td>
<td>days</td>
<td>0.57</td>
<td>0.55</td>
<td>0.89</td>
<td>0.96</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>DHF2</td>
<td>High flow duration 2</td>
<td>days</td>
<td>2.26</td>
<td>1.66</td>
<td>2.80</td>
<td>3.13</td>
<td>2.49</td>
<td></td>
</tr>
<tr>
<td>DHF3</td>
<td>High flow duration 3</td>
<td>days</td>
<td>3.25</td>
<td>3.91</td>
<td>5.11</td>
<td>6.53</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>DHF4</td>
<td>High flow duration 4</td>
<td>days</td>
<td>0.45</td>
<td>0.42</td>
<td>0.70</td>
<td>0.70</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rate of change in flow events</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>days</td>
<td>0.78</td>
<td>0.72</td>
<td>1.27</td>
<td>1.56</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>DHF5</td>
<td>High flow duration 5</td>
<td>m³s⁻¹</td>
<td>-d⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RRM</td>
<td>Rise rate</td>
<td>m³s⁻¹</td>
<td>-d⁻¹</td>
<td>0.14</td>
<td>0.13</td>
<td>0.24</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>RRV</td>
<td>Variability in rise rate</td>
<td>m³s⁻¹</td>
<td>-d⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFM</td>
<td>Fall rate</td>
<td>m³s⁻¹</td>
<td>-d⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFV</td>
<td>Variability in fall rate</td>
<td>m³s⁻¹</td>
<td>-d⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD0</td>
<td>Number of day rises</td>
<td>m³s⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCF1</td>
<td>Change of flow 1</td>
<td>m³s⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCF2</td>
<td>Change of flow 2</td>
<td>m³s⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table above provides data for various flow-related metrics, including high flow duration, rates of change, variabilities, and number of day rises. The units for rise and fall rates are m³s⁻¹d⁻¹, and the variabilities are expressed as m³s⁻¹. The table lists values for different time periods, with the last column showing the variability in rise and fall rates.
Appendix 2. Ordination from the principal component analysis of 108 streamflow variables for the WCC. Standardized PCA based on correlation matrix was obtained by centering and standardization by ‘species’ (in CANOCO 4.5) since ‘species’ were measured in different units. Some of the data points were jittered (where overlapping occurred) to improve clarity.