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Bacterioplankton biogeography of the Mississippi river basin

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BACTERIOPLANKTON BIOGEOGRAPHY OF THE MISSISSIPPI RIVER BASIN

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ABSTRACT

Bacterioplankton are important structural and functional components of river networks, yet their biogeographical patterns in these systems are largely unknown. Using Illumina sequencing of the 16S rRNA gene, we characterized variation in bacterioplankton community alpha diversity (within-sample richness of operational taxonomic units, OTUs) and beta diversity (between-sample differences in composition) (1) along a 1,300-km downstream reach of the Mississippi River, (2) over a range of temporal scales in the Lower Mississippi River (LMR), and (3) along cross-sectional transects repeated monthly for six months across the LMR floodplain. Particle-associated assemblages were richer in bacterial OTUs and more productive than free-living assemblages, indicating the importance of suspended particles as microhabitats for bacterial diversity and activity. Particle-associated richness steadily increased downstream along the river, suggesting greater niche availability, and/or colonization of particles with downriver flow. Communities tended to shift abruptly in composition after converging with major tributaries of the network (i.e. the Illinois, Missouri, and Ohio rivers), however there was an additional but transient shift at Memphis, Tennessee. Conversely, composition changed more gradually within stretches between these points.

In the LMR, there was high variation in alpha diversity over daily and weekly timescales. In contrast, there was successional change in beta diversity that corresponded to temporal trends in water temperature, dissolved nitrogen and chlorophyll *a*. Particle-associated productivity aligned almost exclusively with temperature. Results indicate that community richness, composition, and productivity varied at discrete timescales in the LMR in response to different sets of environmental drivers. Across the LMR floodplain, variation in communities corresponded to the degree and timing of hydrologic connectivity to the main river channel. During high water, floodplain community composition was similar to main channel assemblages. Following hydrologic disconnection, floodplain communities were dominated by cyanobacteria and distinct from the river. After prolonged disconnection (4-5 months), there was convergence in composition of floodplain and river communities which were dominated by heterotrophic taxa. Results suggest that succession of floodplain communities was initiated by river inputs at high river stages. In summary, bacterioplankton community composition and productivity of the Mississippi River Basin varied over space and time which corresponded strongly to environmental variability.

DEDICATION

For my grandparents and Spring. I miss you all very much.

LIST OF ABBREVIATIONS AND SYMBOLS

CHLA	Chlorophyll *a*
DBRDA	Distance-based redundancy analysis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
HG	Helena gage
LML	Lower Mellwood Lake
LMR	Lower Mississippi River
MDL	Middle DeSoto Lake
MML	Middle Mellwood Lake
MMR	Middle Mississippi River
MOL	Moon Lake
MRB	Mississippi River Basin
NMDS	Non-metric multidimensional scaling
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
OUT	Oxbow lake outflow
PN	Particulate nitrogen
POC	Particulate carbon
PCA	Principal components analysis

rkm	River kilometer
RMC	River main channel
rRNA	Ribosomal ribonucleic acid
SBP	Sandbar pool
TDOC	Total dissolved organic carbon
TDN	Total dissolved nitrogen
TEMP	Water temperature
TSS	Total suspended solids
TURB	Turbidity
UMR	Upper Mississippi River
UMMC	University of Mississippi Medical Center
WDP	Wingdam pool

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CHAPTER I: INTRODUCTION

1.1 Background

Biogeography is a scientific discipline that aims to describe patterns in the distribution of biodiversity, and identify causal mechanisms that structure communities in space and over time (Lomolino et al. 2006). Organisms are not randomly distributed around the world, and assemblages seem to be structured by the combination of contemporary interactions among community members and their environment, and historical events (e.g. past environmental conditions, and dispersal and extinction of species; Lomolino et al. 2006). Biogeography of conspicuous plants and animals has been an area of study for centuries. With the advent of 16S rRNA-based molecular techniques in recent decades, it is now possible to analyze the biodiversity and distribution of bacteria and other microorganisms (Head et al. 1998, Dorigo et al. 2005, Zinger et al. 2012). As for plant and animal communities, it is now known that bacteria can exhibit biogeographic patterns (Fierer and Jackson 2006, Martiny et al. 2006, Fierer et al. 2007, Fierer 2008, Fierer and Lennon 2011).

A long-held hypothesis applied to bacterial biogeography is that "everything is everywhere, but, the environment selects" (Baas-Becking 1934). This hypothesis is based on bacteria being abundant (Whitman et al. 1998), prolific reproducers, and able to disperse easily due to their small size (Finlay 2002). However, several studies have found

that geographic distance was related to the distribution of bacterial taxa among habitats with similar habitat characteristics (Cho and Teidje 2000, Papke et al. 2003, Reche et al. 2005, Ghiglione et al. 2012), indicating that both dispersal limitation and environmental selection can contribute to bacterial biogeographic patterns (Martiny et al. 2006, Nemergut et al. 2010, Lindström and Langenheder 2012). Thus, the processes that shape the distribution of larger organisms may also help elucidate the mechanisms that determine biogeographic patterns of bacteria (Martiny et al. 2006). Within this framework, Martiny et al. (2006) proposed four alternative hypotheses for the spatial distribution of bacterial communities: (i) microbial taxa are randomly distributed (i.e. the null hypothesis), (ii) environmental selection alone shapes bacterial communities (i.e. the Baas-Becking hypothesis), (iii) dispersal limitation alone shapes bacterial communities, or (iv) a combination of environmental selection and dispersal limitation shape bacterial communities. However, the relative importance of contemporary environmental conditions, past environmental conditions, and dispersal limitation in controlling the spatial distribution of bacterial communities is dependent on the type of environment (Lindström and Langenheder 2012).

Large river networks are ideal ecosystems to test the interplay of past and current events that determine biogeographic patterns of bacterial communities. The Mississippi River Basin (MRB) is a large river network that includes the Arkansas River, Missouri River, Ohio River, and the Lower and Upper Mississippi River (Brown et al. 2005). These rivers are influenced by the geomorphologic structure of the basins in which they flow, and anthropogenic land uses shaping their chemical and physical properties

(Goolsby et al. 1999, Turner and Rabalais 2004). As a consequence, these rivers and their respective basins support distinct, diverse, and sometimes predictable macro-organism assemblages based on spatial (e.g., local and regional environmental differences) and temporal river conditions such as discharge (Muneepeerakul et al. 2008).

In large river environments, bacteria are abundant and potentially diverse. In the turbulent water column of a river, planktonic bacteria (bacterioplankton) occur as free-living cells, attached to particles, or as assemblages of a few cells (Ochs 2003). Free-living cells and those associated with particles are different communities from one another (Crump et al. 1998, Besemer et al. 2005, Jackson et al. 2014). Bacterioplankton are important as a numerically-large (typically ~ 1 x 10^6 cells per milliliter or more) component of aquatic food webs (Whitman et al. 1998). In addition, because as a group bacterioplankton possess diverse metabolic capabilities with respect to energy transduction and nutrient use, and turnover rapidly, these organisms are particularly important in catalyzing biogeochemical transformations in river ecosystems (Ochs 2003). Thus, it is important to describe bacterioplankton communities and their physiological aspects to better understand how these ecosystems function in terms of biogeochemical processes (del Giorgio and Pace 2008) and trophic structure. However, little is known about the dynamics of bacterioplankton diversity and distribution in large river systems and what these organisms contribute to these ecosystems in terms of metabolically-significant processes.

What, then, are the local and regional environmental factors that shape bacterial assemblages in the MRB? Furthermore, as these environmental conditions vary over

time, at what time frames do bacterial assemblages and functions vary as well? Also, are there endemic bacterioplankton communities in the MRB, or are all assemblages the same across all rivers/habitats? My research project involved three components aimed to determine bacterial community composition, functional attributes, and biogeographic patterns in the main channels of major rivers in the MRB, and across the Lower Mississippi River (LMR) floodplain: (1) A study took place along a downstream transect of the main axis of the Mississippi River and its major tributaries. This "downstream study" analyzed the MRB ecosystem as a whole and analyzed regional as well as local environmental conditions that determine distributions of bacterioplankton communities. (2) A "temporal study" took place at a single site on the LMR over one year. This component determined variation in bacterioplankton community composition and productivity over brief and long timescales. (3) A study took place across a cross-sectional transect of the floodplain of the LMR. This "floodplain study" allowed for an expanded view of the structure of bacterial communities across diverse aquatic habitats in the LMR floodplain.

1.2 Downstream Study

Large rivers change in hydrology, geomorphology, organic matter and chemical composition along the basins in which they flow and these factors create habitat heterogeneity and shape biocomplexity (Thorp et al. 2006). For example, confluences of smaller tributaries and the main stem can be zones where mixing of the two fluvial systems produces a combination of habitats for organisms to occupy (Benda et al. 2004). As a large river is developing from headwaters to mouth, many smaller tributaries

contribute to the growing river system by adding unique chemical and physical features creating habitat complexity.

Only a few studies have investigated variation in bacterioplankton community structure across a large river system and the factors that influence this variation. Winter et al. (2007) found that bacterial diversity increased longitudinally from headwaters to the mouth of the Danube River and environmental factors influenced richness. Bacterioplankton diversity was positively correlated with phosphate and negatively correlated with chlorophyll *a* concentration. Conversely, Sekiguchi et al. (2002) observed a decrease in bacterial diversity from upstream to downstream in the Changjiang River and found no effect of environmental factors controlling bacterioplankton assemblages.

The MRB, a collection of unique sub-basins and their rivers, covers around 14% of North America and drains nearly 42% of the contiguous United States (Brown et al. 2005). These sub-basins vary in climatic conditions, physiography, and anthropogenic land uses resulting in differences in suspended sediment, carbon, nitrogen, and phosphorus loads of their rivers (Goolsby et al. 1999, Turner and Rabalais 2004). Given the environmental heterogeneity among rivers of the MRB, are there distinct bacterioplankton assemblages in each river? Furthermore, when these rivers meet, are there concurrent changes in assemblage diversity and composition?

The objective of this study was to assess evidence for bacterioplankton community structure in large rivers of the MRB and, if detected, identify factors that control bacterioplankton community composition before and after confluences of major tributaries. I hypothesized that each tributary, having unique chemical (e.g., nutrient

composition) and physical properties (e.g., suspended solid loads), would have different bacterial assemblages. After convergences of tributaries, assemblages would mix causing a dramatic change in bacterioplankton diversity. As physiochemical conditions stabilize downstream after confluences, there would be a more gradual change in assemblage composition. These hypotheses suggest that the dominant influence on community composition in these rivers is environmentally-mediated and not due to constrained dispersal. The null hypothesis was that bacterial community composition among samples from all rivers would not differ. This situation would arise if bacterioplankton are able to disperse across the MRB, and physiochemical aspects of each river did not influence community composition.

This study was designed to determine whether bacterioplankton assemblages vary over regional (between basins) and local (river confluences) spatial scales. The MRB is an excellent system to test these hypotheses because of the structure of this large river network. I sampled 13 locations along the Mississippi River and one location from each major tributary including the Illinois, Missouri, Ohio, Arkansas, and Yazoo Rivers over a two-week period (23-July – 3-August) in 2013. Samples were collected in tandem just above the confluence of the Mississippi River and its tributaries and at 100 – 150 km increments of distance per day along the Mississippi River. Because sampling took place during a specific two-week period, it may not be possible to extrapolate results of this study to longer timescales over which bacterioplankton communities vary in large river systems. For this reason, I conducted a study to determine the timescales over which communities change at a single location in the river.

1.3 Temporal Study

Large river ecosystems can change temporally in flow/discharge (Poff et al. 1997), chemical composition (Goolsby et al. 1999), and in sources/types of organic matter (del Giorgio and Pace 2008). These factors have been suggested as determining river bacterioplankton community structure. Crump et al. (2009) measured temporal patterns of bacterioplankton diversity in six large rivers of the arctic circumpolar region. They found that communities among rivers were similar to each other, co-varied from season to season, and community composition could be predicted depending on seasonal flow and biochemical regimes accompanied by seasonal changes. Similarly, bacterial productivity has been related changes in dissolved organic matter and nutrient concentrations, sediment load, and other properties of aquatic environments (Judd et al. 2006), including large river systems (Besemer et al. 2009, Ochs et al. 2010).

The LMR transports large amounts of dissolved and particulate inorganic and organic materials to the Gulf of Mexico with an average annual discharge around 17,000 m^3 sec^{-1} (Turner and Rabalais 2003). The water of the LMR is a collection of discharges from its tributaries, which can differ greatly in suspended sediment and nutrient loads (Turner and Rabalais 2004). The LMR has its own unique drainage basin made of smaller rivers (Brown et al. 2005), but is also influenced by major tributary rivers including the Arkansas River, Missouri River, Ohio River, Tennessee River, and Upper Mississippi River (Brown et al. 2005). What environmental factors occurring in what time frames determine bacterioplankton assemblages and productivity in the LMR?

The objective of this study was to analyze bacterioplankton assemblages and productivity at a single site on the LMR near Tunica, Mississippi at daily, weekly, and monthly time intervals. I hypothesized that bacterioplankton community composition and productivity would display more variation between samples taken at longer timescales (i.e. between months, cf. between days), and that this variation would be a response to temporal changes in physical conditions and chemical composition.

This study was designed to determine the variability of bacterioplankton assemblages and functional attributes over brief and long timescales in the LMR. A site in the main channel near Mhoon Landing, Tunica, Mississippi was chosen for the temporal study. Temporal sampling included three phases. First, samples were collected once a month from February 2013 to January 2014 for a total of 12 monthly samples. Second, samples were collected weekly from 3-June to 15-July 2013 for a total of seven weekly samples. Third, samples were collected daily from 24-June to 1-July for a total of eight daily samples. This study was useful to evaluate the timescales of community variation for comparisons to the downstream and floodplain studies. Given that the suspended sediment and chemical loads of the LMR have been well-documented over decades (Turner and Rabalais 2004) it may be reasonable to extrapolate results of this study for years with similar physiochemical conditions at this site.

1.4 Floodplain study

Large rivers have areas of diverse geomorphologies that include the river main channel, slack-water areas, and backwater lakes that are collectively part of the floodplain

system (Junk et al. 1989, Baker et al. 1991). These floodplain habitats often differ from the main channel in chemistry (Schramm et al. 2009), hydrology, other physical characteristics (Fremling 2005), and biota (Amoros and Bornette 2002) especially during low water periods when disconnected from the main channel (Pongruktham and Ochs 2015). The Riverine Ecosystem Synthesis (Thorp et al. 2006) described river systems comprised of many different patches based on hydrology and geomorphology and these patches determine community structure and function. In the Danube river-floodplain system, Besemer et al. (2005) found that river connectivity structured environmental conditions and bacterial communities within frequently inundated floodplain pools, whereas temporal variation in the environment and bacterial communities of isolated pools was associated with seasonal dynamics. In addition, particle-associated and free-living assemblages differed significantly from each other in composition and correlated with different environmental variables. Specifically, the particle-associated component was more heterogeneous compared to free-living assemblages, and correlated with fluxes in algal biomass and terrestrial organic matter.

Large rivers like the LMR have floodplains (Sedell et al. 1989) and riparian zones (Thorp and Delong 1994) as important contributors of nutrients and habitat diversity. Even though the LMR is highly regulated with levees reducing its floodplain, there are many habitat types including the river main channel, slack-waters (along the main channel), and backwater sloughs and oxbow lakes (Baker et al. 1991). Furthermore, depending on river stage, these habitats are connected to a varying degree. How do

chemical and physical variation, habitat heterogeneity, and changing connectivity correlate with temporal and spatial distributions of bacterioplankton?

The objective of this study was to determine the temporal and spatial scales over which assemblages of bacterioplankton change in different local habitats of the LMR floodplain, and to identify the variables that drive those changes based on the degree of connectivity the main channel has with the floodplain. I hypothesized that during periods of high connectivity, communities among the main channel and floodplain habitats would be similar in composition to each other due to influences of main channel inputs. These inputs would be opportunities for main channel assemblages to be introduced to the floodplain, along with their environments (e.g. the habitat suitable for those assemblages' needs). Furthermore, I hypothesized that during disconnected periods, there would be divergence in bacterioplankton communities across different floodplain sites as environmental conditions diverged from the river. Factors such as turbidity (Besemer 2005), chlorophyll *a* and nutrient concentrations (Docherty et al. 2006) would be major determinants of these new community compositions. The null hypothesis was that bacterial community composition among all floodplain habitats would not differ from main channel assemblages. This situation would arise if bacterioplankton were able to disperse across the floodplain, and once dispersed local physiochemical aspects of floodplain sites did not influence community development.

This study was designed to investigate temporal patterns of distribution in bacterial community composition across the LMR floodplain, and how main channel connectivity with floodplain habitats determines that distribution. To address this

question, I sampled eight sites that vary in connectivity with the LMR main channel depending on the river stage. Sites were sampled beginning with high water periods of the LMR during late spring/summer, and extended through low water periods during mid-late fall. One site was sampled in the well-mixed main channel. Three sites that flank the main channel were sampled including an oxbow lake outflow site, a site at the downstream direction behind a wingdam, and a sandbar pool. Two oxbow lakes in the current LMR floodplain were sampled including two sites on Mellwood Lake, and one site on Desoto Lake, Mississippi. The aforementioned sites are on an approximately east-west transect across the river floodplain. Also, one site, Moon Lake, was sampled at an oxbow lake outside of the extant LMR floodplain, and this site served as an approximate control system.

1.5 Significance

The goal of this dissertation was to evaluate bacterioplankton assemblage diversity, biogeographic patterns, and productivity in an under-studied large river ecosystem. Little is known about bacterioplankton community structure and functional attributes in the MRB. Because these organisms are the most abundant living component of aquatic ecosystems, and perform diverse biogeochemical functions, it is important to describe bacterioplankton attributes to better understand how these ecosystems are structured and function ecologically. This project addressed questions about how bacterioplankton communities vary structurally over broad and short spatial scales and how bacterial productivity varies in a large river system over a range of temporal scales.

Do bacterioplankton community structures vary among rivers in the MRB with unique chemical and physical properties? What happens to the Mississippi River microbiome when rivers meet? The downstream study not only showed that bacterioplankton assemblages vary among basins of the MRB, but also determined where and how the Mississippi River microbiome may shift in composition along the length of the river.

What environmental factors occurring over what timescales determine bacterioplankton assemblages and productivity in the LMR? The temporal study elucidated the various timescales over which bacterioplankton assemblage structure and productivity changed in this large river ecosystem, which aligned with the temporal scales of variation in distinct environmental factors.

How do chemical and physical variation, habitat heterogeneity, and changing connectivity correlate with spatial and temporal distributions of bacterioplankton? The LMR floodplain is an important area of diverse habitats, but also plays a role as a source and sink of nutrients that continually shift due to annual flow regimes (Ochs and Shields, *in press*). This floodplain may be important to the regulation of nitrogen and phosphorus that contribute to the hypoxic zone in the Gulf of Mexico. The floodplain study elucidated bacterioplankton diversity over a wide range of diverse habitats across the LMR floodplain, and provided insights regarding the role of main channel connectivity in shaping assemblage composition among floodplain habitats of the LMR.

The combination of these three studies presents a uniquely comprehensive view of bacterioplankton biogeography in the MRB. Furthermore, by elucidating

bacterioplankton productivity over a range of temporal scales in the LMR this study furthers our understanding of how large river ecosystems are functioning over relatively long and short timescales.

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CHAPTER II:

PATTERNS OF VARIATION IN DIVERSITY OF THE MISSISSIPPI RIVER MICROBIOME OVER 1,300 KILOMETERS

Abstract

We examined the downriver patterns of variation in taxonomic diversity of the Mississippi River bacterioplankton microbiome along 1,300 river kilometers, or approximately one third the total length of the river. The study section included portions of the Upper, Middle, and Lower Mississippi River, confluences with five tributaries draining distinct sub-basins, river cities, and extended stretches without major inputs to the Mississippi. The composition and proportional abundance of dominant bacterial phyla was distinct for free-living and particle-associated cells, and constant along the entire reach, except for a substantial but transient disturbance near the city of Memphis, Tennessee. At a finer scale of taxonomic resolution (operational taxonomic units, OTUs), however, there were notable patterns in downriver variation in bacterial community alpha diversity (richness within a site) and beta diversity (variation in composition among sites). There was a strong and steady increase downriver in alpha diversity of OTUs on suspended particles, suggesting an increase in particle niche heterogeneity, and/or particle colonization. Relatively large shifts in beta diversity of free-living and particle-associated communities occurred following major tributary confluences and transiently at Memphis, while in long stretches between these points diversity typically varied more gradually.

We conclude that the Mississippi River possesses a bacterioplankton microbiome distinct in diversity from other large river microbiomes in the Mississippi River Basin, that at major river confluences or urban point sources its OTU diversity may shift abruptly and substantially, presumably by immigration of distinct external microbiomes, but that where environmental conditions are more stable along the downriver gradient, microbiome diversity tends to vary gradually, presumably by a process of successional change in community composition.

2.1 Introduction

All ecosystems have a microbial community, or microbiome, from the tissues and organs of individual animal and plant hosts (Costello et al. 2012, Christian et al. 2015), to aquatic or terrestrial environments (Gibbons and Gilbert 2015). The microbiome is involved with essential ecosystem processes whether it is contributing to host metabolism (Turnbaugh et al. 2007, Human Microbiome Project Consortium 2012, Borer et al. 2013) or biogeochemical cycling of nutrients at regional or global spatial scales (Falkowski et al. 2008). Hence, variation in microbiome composition may have implications for the functional attributes, services, or adaptability of the ecosystem (Blaser et al. 2016).

A fluid ecosystem may exhibit change in microbiome composition from one region to another along the path of flow. Examples of such systems include the human gastrointestinal tract (Walter and Ley 2011) and lotic systems such as streams or rivers (Crump et al. 2007, Savio et al. 2015). Variation in diversity of the microbiome along the flow path may be slow and gradual along regions of weak gradients of environmental change and selection, or occur abruptly at nexuses with other ecosystems (Sekiguchi et

al. 2002, Winter et al. 2007, Jackson et al. 2014). Examination of microbiome diversity along a fluid ecosystem having both major confluences, and long stretches of slow, gradual change between these interfaces, may reveal the relative importance of abrupt supplementation of distinct, external microbiomes, versus adaptation and gradual selection moving downriver.

The main objective of this study was to determine the spatiotemporal pattern of variation in planktonic microbial community diversity of the Mississippi River as it flows downriver over 1,300 river kilometers (rkm), converging intermittently with other rivers that drain large sub-basins of the Mississippi River Basin. We hypothesized that changes in the diversity of the microbiome would be relatively large and abrupt following mixing at confluences, but more gradual with increasing downriver distance from confluences. This hypothesis was predicated on a prior finding that each of these rivers has a distinct microbiome (Jackson et al. 2014); the repeatability of this finding and its implications were tested independently in this study. By following microbiome diversity with river flow from directly above, to close below, to far between, and within tributary inputs we were able to evaluate the likelihood of these two proposed mechanisms, sudden tributary inoculation vs. gradual environmental selection (Leibold et al. 2004, Savio et al. 2015, Staley et al. 2015), on the pattern of downriver variation in compositional diversity of the Mississippi River microbiome.

For analysis of the microbiomes of the Mississippi River and its tributaries, we utilized barcoded next generation Illumina sequencing of the bacterial 16S rRNA gene (Kozich et al. 2013). This approach facilitates detailed characterization of microbiome

composition along temporal and spatial gradients. When viewed at a low level of taxonomic resolution a microbiome may appear fairly homogenous among ecosystems having broadly shared environmental characteristics (Lozupone and Knight 2007, Lauber et al. 2009, Nemergut et al. 2010, Cho and Blaser 2012). However, when viewed at a high level of taxonomic resolution, shifts in microbiome composition may become evident with consequences for microbiome function (Staley et al. 2013). Here we compare downriver spatial variation in the microbiome(s) at a low level of taxonomic resolution, phylum-level classification of bacterial OTUs (operational taxonomic units); and at a high level of resolution, individual bacterial OTUs.

2.2 Methods

2.2.1 The Mississippi River system

The Mississippi River Basin encompasses 41% of the lower United States and is composed of six major sub-basins, including the Upper Mississippi River Basin, the Missouri River Basin, the Ohio/Tennessee River Basin, the Arkansas/White/Red River Basin, and the Lower Mississippi River Basin. Each sub-basin is distinct in land-use practices, physiography, climate, and the physicochemical properties of its major rivers (Goolsby et al. 1999, Turner and Rabalais 2004). The 3,700 rkm Mississippi River itself can be divided into three sections based on where it merges with its two largest tributaries The Upper Mississippi River (UMR) has its headwaters at Lake Itasca, Minnesota, and ends at the confluence with the Missouri River, at St. Louis, Missouri. The latter 1,059 rkm of the UMR section consists of a series of large pools created by navigation dams. The Middle Mississippi River (MMR) is unimpounded and free-flowing and extends

about 310 rkm from the Missouri River confluence to the confluence with the Ohio River, at Cairo, Illinois. The Lower Mississippi River (LMR) is also free-flowing and extends 1,600 rkm from the Ohio River confluence to its outlet into the Gulf of Mexico.

2.2.2 Sampling the Mississippi River Network

Water samples were collected in sequence from a series of thirteen sample sites on the Mississippi River extending from just above St. Louis, Missouri to Natchez, Mississippi, a distance of 1,300 rkm. Additionally, samples were collected from the mouths of five tributaries to the Mississippi, each of which drains an important sub-basin. Only one site per day was sampled on the Mississippi starting on July 22, 2013 at the north-most site (site 1) and ending on August 03, 2013 (site 13) (Table 2.1; Figure 2.1). Sample site 1 was located in pool 26 of the UMR. All other sample sites were in unimpounded, free-flowing portions of the Mississippi or the tributaries.

Table 1. Sites sampled on the Mississippi River and its major tributaries, July-August 2013. [a] = UMR, Upper Mississippi River; MMR, Middle Mississippi River; LMR, Lower Mississippi River. [b] = Sample sites on the Mississippi River are numbered sequentially from north to south. [c] = rkm, river kilometers. rkm for sites on the Mississippi River starting from Head of Passes, Louisiana, which is rkm 0. [d] = Discharge data by date provided by the U.S. Geological Survey and the U.S. Army Corp of Engineers.

		Mississippi River				
Date	River[a]	Site[b]	rkm[c]	Location	Discharge (m^3/s)[d]	Depth (m)
7/22	UMR	1	1,889	N 38 56.788, W 90 28.497	1,880	3.4
7/22	Illinois			N 38 57.897, W 90 29.778	435	6.7
7/23	UMR	2	1,851	N 38 49.688, W 90 06.685	2,315	7.6
7/23	Missouri			N 38 49.587, W 90 08.054	1,479	4.8
7/24	MMR	3	1,733	N 38 00.196, W 90 02.823	3,794	9.1
7/25	MMR	4	1,617	N 37 20.077, W 89 28.985	6,098	8.8
7/26	MMR	5	1,537	N 37 01.596, W 89 12.993	5,737	13.5
7/26	Ohio			N 37 01.542, W 89 10.531	7,584	12.1
7/27	LMR	6	1,432	N 36 34.716, W 89 31.331	13,321	10.1
7/28	LMR	7	1,304	N 35 53.143, W 89 46.106	12,856	15.4
7/29	LMR	8	1,191	N 35 13.442, W 90 04.502	13,960	12.8
7/30	LMR	9	1,107	N 34 44.586, W 90 27.001	12,912	16.2
7/31	LMR	10	941	N 33 49.000, W 91 02.902	14,753	12.3
7/31	Arkansas			N 33 48.615, W 91 06.538	1,017	5.9
8/01	LMR	11	803	N 32 54.010, W 91 03.972	15,770	18.5
8/02	LMR	12	708	N 32 20.801, W 90 57.772	17,273	12.5
8/02	Yazoo			N 32 23.352, W 90 54.981	142	11.9
8/03	LMR	13	587	N 31 33.642, W 91 24.596	17,415	22.8

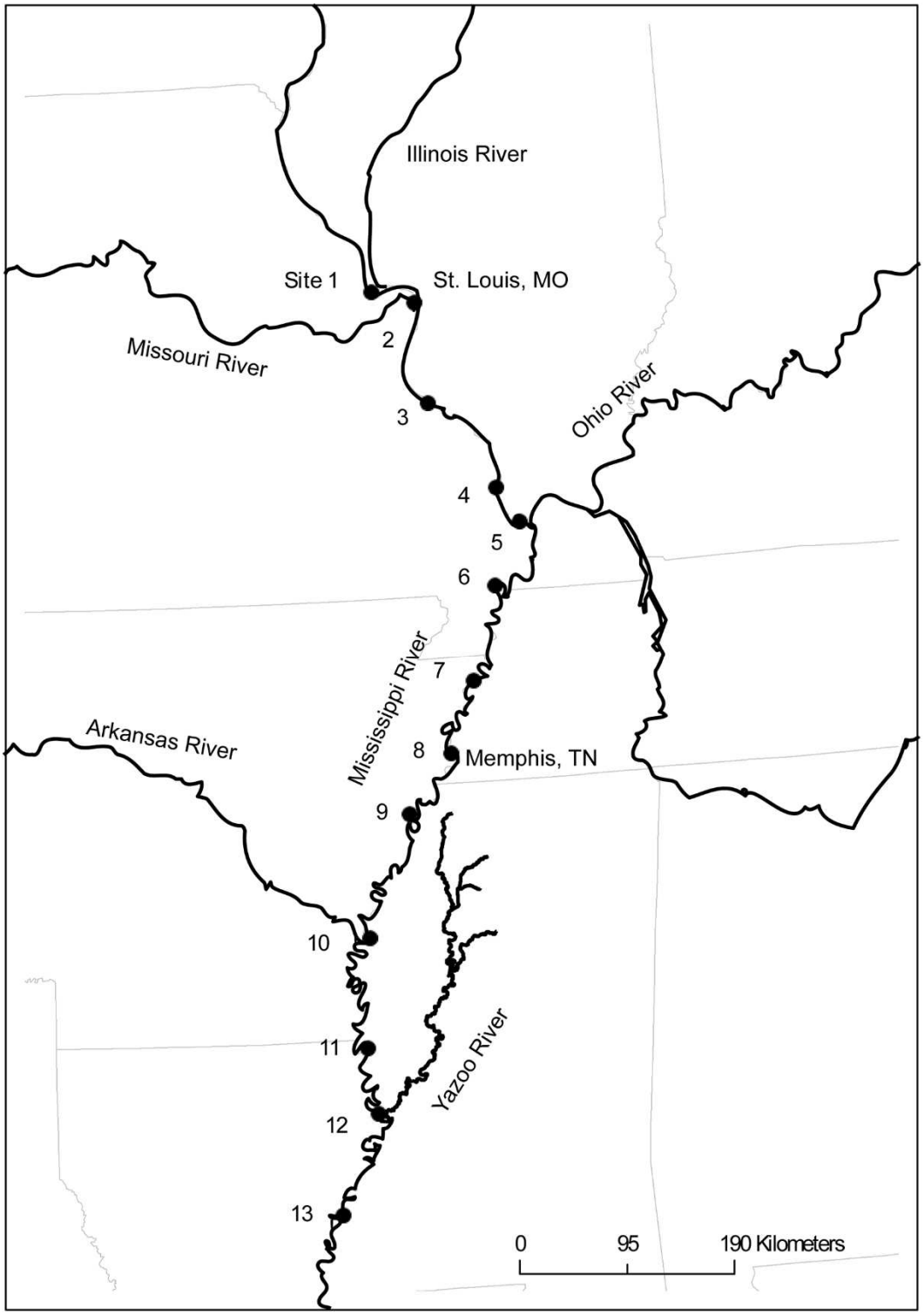

Figure 1. Sample sites on the Mississippi River. Sample sites are indicated by closed circles and referred to by number, from site 1 to site 13, in order from north to south (Table 2.1).

The daily sampling regime was designed to roughly keep up with river flow velocity, estimated during our sample period in our sampling reach at 100 – 150 km per day. This estimate is based on continuous measurements made by the United States Geological Survey at Belle Chasse, Louisiana, (https://waterdata.usgs.gov/nwis/uv?site_no=07374525), adjusted for a river velocity of about 1.6 km/h faster above the floodgate system of the Old River Control Structure (personal communication, J. Ruskey). With the exception of the first two sample sites on the UMR, sites 1 and 2, all sample sites were separated by >80 rkm (ranging from 80 to 166 rkm; mean = 116 rkm) (Table 2.1).

Of the thirteen sample sites on the Mississippi, five sites were located within 3-8 rkm above confluences with the five river tributaries. Within 1 h of the time that these sites were sampled, the associated tributary was also sampled within its mouth just above the confluence. Sample sites on the Mississippi subsequent to tributary inputs were located 34 to 130 rkm below confluences to allow for full mixing of the convergent rivers.

Sampled tributaries include three that are relatively large in the percentage of water they add to the Mississippi at their confluence, and two that are relatively small in this percentage. The former tributaries are the Illinois River (contributing 19% of the summed discharge), the Missouri River (contributing 39%), and the Ohio River (contributing 57%). The two relatively small tributaries are the Arkansas River (contributing 7%), and the Yazoo River (contributing 1%) (Table 2.1). Henceforth, where

we refer to *major* tributaries, we are referring specifically to the relatively large tributaries: the Illinois River, the Missouri River, and/or the Ohio River.

Samples were collected from 0.5 m depth at mid-river locations. River depths at sample sites (Table 2.1) were measured using a hand-held Hawkeye H22PX digital sonar system. All samples were collected between 10:00 and 13:00 h, except for on July 26 at the Middle Mississippi/Ohio confluence where collections were made between 16:00-16:30 h. Each collection consisted of three sterilized 1-L Nalgene bottles for determination of water chemical, physical, and biological parameters, and three sterilized 500-mL Nalgene bottles for determination of bacterial community structure. Each sample bottle was rinsed three times with river water prior to being filled with sample. Samples were stored in river water in insulated coolers to maintain ambient temperature during transportation (1-2 h) before being processed. All samples used in this study were collected from public river waterways for which permission to obtain samples was not required.

2.2.3 Water parameter measurements

For chemical analyses, water samples (100- or 200-mL) were filtered through ashed 47-mm diameter Whatman GF/F filters. Filtered sample water and filters were frozen immediately for subsequent chemical analyses. Orthophosphate, nitrate, and ammonium, were measured from filtered water following standard colorimetric methods (Wetzel and Likens 2000), and total dissolved nitrogen and total dissolved organic carbon were measured using a Shimadzu TOC-L Total Organic Carbon analyzer. Particulate carbon and nitrogen were measured from filters concurrently by dynamic combustion

using a Perkin Elmer 2400 Series II CHNS/O analyzer, and total suspended solids concentration was measured by gravimetry. Chlorophyll *a* was extracted from filters in 90% NH_4OH-buffered acetone for 24 h at 5°C and measured by spectrophotometry (Wetzel and Likens 2000). Water temperature and pH were measured on-site using a YSI Professional Plus multiparameter meter

2.2.4 DNA extraction and sequencing

For analysis of bacterial community composition, the 500-ml sample bottles were mixed, and sub-samples (100 mL per bottle) withdrawn. These sub-samples were filtered in series through a sterile Millipore 3-µm pore-size polycarbonate filter to collect particle-associated or relatively large bacteria, and a sterile Millipore 0.22-µm pore-size polyethersulfone filter to collect free-living bacteria (Jackson et al. 2014, Savio et al. 2015). Each filtration was performed at <5 mm Hg and filters were kept at -20°C until DNA extraction.

PowerWater DNA isolation kits (MoBio, Carlsbad, California) were used to extract DNA from each filter. The V4 region of 16S rRNA genes was amplified using forward (5'-GTGCCAGCMGCCGCGGTAA) and reverse (5'-GGACTACHVGGGTWTCTAAT) primers coupled with dual index barcodes that are optimized for Illumina MiSeq sequencing (Kozich et al. 2013). Amplifications were run under conditions defined previously (Stone and Jackson 2016). Briefly, DNA was initially denatured at 95°C for 2 min, then run through 30 cycles of denaturation (95°C for 20 s), annealing (55°C for 15 s), and elongation (72°C for 2 min), and ended with a final elongation step (72°C for 10 min). Amplified DNA was normalized by sample using

SequalPrep Normalization Plates (Life Technologies, Grand Island, New York), pooled, and sequenced by the Molecular and Genomics Core Facility at the University of Mississippi Medical Center using an Illumina MiSeq. Sequence data were processed using *mothur* following guidelines set forth by Schloss et al. (2009) and Kozich et al. (2013).

Sequences were aligned to reference V4 sequences using the SILVA rRNA database (Pruesse et al. 2007) and sequences that did not align with the V4 region and homopolymers >8 bp were discarded. Prior to classification, sequences nearly identical (<2 bp differences) to one another were merged (Stone and Jackson 2016), and potential chimeras identified using UCHIME (Edgar et al. 2011) and removed. Taxonomic classification of sequences was performed using the Greengenes database (DeSantis et al. 2006), a database more suitable for classifying 16S rRNA sequences than the SILVA database used to align sequences (Werner et al. 2012). Any sequence classified as being non-bacterial in origin (e.g. sequences derived from chloroplasts, mitochondria, Archaea, Eukarya, or unknown sources) was removed. All bacterial sequence reads were clustered into OTUs based on ≥97% similarity and identified to the highest possible taxonomic resolution. All sequences were deposited in the NCBI SRA database under the BioProject ID PRJNA356973.

2.2.5 Sequence analyses

Sequence data were visualized and analyzed using the *phyloseq* version 1.14.0 (McMurdie and Holmes 2013), *vegan* version 2.4-1 (Oksanen et al. 2016), and *ggplot2* version 2.1.0 (Wickham 2016) packages implemented in R version 3.2.4 revised (R Core

Team 2013). Two free-living bacterial samples obtained from the Mississippi River were removed from the data set because of poor sequencing depth (<5,000 reads per sample) (Lundin et al. 2012).

Patterns at the phylum-level in downriver community composition were examined using average relative abundances of OTU-reads grouped by phylum or "unclassified" for OTUs classified to domain Bacteria only. Average relative abundances were calculated from replicate samples for each site on the Mississippi. Singleton and other rare OTUs represented by reads that comprised <0.001% of total reads were removed to reduce inclusion of potentially erroneous sequence data into analyses.

For analysis of beta diversity, sample sequences were normalized to provide the same number of reads per sample. Before normalization, rare OTUs, defined as above, were removed. Then, each sample of bacterial sequences was randomly subsampled to equal sequencing depths of samples having the fewest number of reads. From this process we obtained for all rivers, 6,603 and 9,617 reads per sample, for free-living and particle-associated bacteria, respectively. For the Mississippi River alone, we obtained 6,587 and 11,581 reads per sample, for free-living and particle-associated bacteria, respectively. To quantify beta diversity, we used the Bray-Curtis dissimilarity index that compares proportional abundances of OTUs among samples. To visualize patterns in beta diversity, dissimilarity matrices were ordinated by non-metric multidimensional scaling (NMDS). Dissimilarity matrices were analyzed statistically by permutational multivariate analysis of variance (function "adonis" in the package *vegan*) to test the null hypothesis of no differences in centroids of communities grouped by river or, for the Mississippi alone, the

river section (Anderson and Walsh 2013). Removal of rare OTUs, sequence-number normalizations, dissimilarity calculations, and ordinations were performed using the functions "prune_taxa", "rarefy_even_depth", "phyloseq::distance", and "ordinate" in the *phyloseq* package, respectively. All random number generator seeds were set to "1000" for standardizations and statistical analyses to ensure that these analytical results were reproducible (McMurdie and Holmes 2013).

To determine downriver patterns in microbiome alpha diversity, the richness of OTUs obtained from untrimmed sequence data (e.g. including singleton and rare OTUs) was analyzed using the "estimate_richness" function in the *phyloseq* package (McMurdie and Holmes 2013). Samples were randomly subsampled (100 iterations) to equal sequencing depths of samples having the fewest number of sequence reads. Free-living and particle-associated bacterial samples were normalized to 6,690 reads per sample, the fewest number of reads among all samples. After subsampling, the number of OTUs was averaged across replicate samples for each of the sites on the Mississippi River and linear regression used to determine the relationships of OTU richness and downriver distance.

2.3 Results

2.3.1 Water parameters

There were distinct differences in chemical, physical, and biological parameters among the three sections of the Mississippi River and its tributaries (Table 2.2). Here, we focus on inputs, and corresponding effects, of the three major tributaries (Illinois, Missouri, Ohio Rivers) to the Mississippi, and on downriver patterns in the Mississippi. Orthophosphate concentration was highest in the Illinois River, contributing to a

concentration increase of 58% between adjacent sites on the UMR, before declining to 83.2 and 74.0 µg/L in the MMR and LMR, respectively. Total dissolved nitrogen, consisting mostly of nitrate, was highest in concentration in the UMR and declined with distance downriver to 3.5 and 1.9 mg/L in the MMR and LMR, respectively. Dissolved organic carbon (TDOC), particulate carbon, and particulate nitrogen also declined from maximum values in the UMR to low and less variable (with the exception of TDOC) values in the LMR. Total suspended solids (TSS) were high in the Missouri, contributing to a 30% increase in TSS from the UMR to the MMR. Following dilution by the Ohio River, TSS declined in the LMR to a mean of 55.6 mg/L (excluding site 9 where TSS was 276 mg/L). Chlorophyll *a* was about 20 µg/L in the UMR, increased to a mean of 26 µg/L after the confluence with the Missouri, then declined to 16.5 µg/L in the LMR. Similar spatial patterns of these parameters in the Mississippi River and its tributaries during summer have been noted in other studies as consequences of sub-basin influences (Goolsby et al. 1999, Turner and Rabalais 2004, Ochs et al. 2010, Jackson et al. 2014).

Table 2. Water parameter measurements from the Mississippi River and its major tributaries, July-August 2013. Values are presented as mean (standard deviation). *n* = number of sites sampled per river or river section, 3 replicates per site. [a] = For the MMR and LMR, parameters are presented as means and standard deviations of site means. For the UMR and tributary rivers, parameters are presented as means and standard deviations of replicates within a site. [b] = Upper Mississippi River sampled above confluence with the Illinois River. [c] = Upper Mississippi River sampled below confluence with the Illinois River. Abbreviations as in text.

Parameter[a]	UMR[b] *n = 1*	UMR[c] *n = 1*	MMR *n = 3*	LMR *n = 8*	Illinois *n = 1*	Missouri *n = 1*	Ohio *n = 1*	Arkansas *n = 1*	Yazoo *n = 1*
PO_4-P µg/L	63.8 (1.3)	101.5 (6.4)	83.2 (6.1)	74.0 (8.8)	154.8 (70.1)	85.4 (8.8)	27.5 (5.2)	18.2 (0.8)	44.7 (4.2)
NO_3-N mg/L	4.0 (0.1)	4.0 (0.0)	3.3 (0.1)	1.7 (0.1)	2.4 (0.0)	1.5 (0.1)	1.0 (0.0)	0.0 (0.0)	0.6 (0.0)
NH_4-N µg/L	24.9 (22.2)	20.2 (1.3)	6.0 (6.6)	18.6 (17.5)	36.8 (9.0)	6.0 (10.4)	11.5 (20.0)	26.2 (2.0)	7.0 (12.1)
TDN mg/L	4.4 (0.1)	4.2 (0.1)	3.5 (0.2)	1.9 (0.2)	3.1 (0.0)	1.6 (0.0)	1.2 (0.0)	0.4 (0.0)	0.8 (0.0)
TDOC mg/L	10.9 (1.0)	9.0 (1.2)	9.5 (1.3)	5.6 (1.2)	8.5 (0.6)	7.3 (0.9)	-	7.7 (0.2)	6.0 (0.1)
pH	8.2	7.7	7.8 (0.0)	7.7 (0.1)	8.1	8.0	7.4	8.0	7.3
POC mg/L	3.2 (0.3)	3.1 (0.1)	3.2 (0.1)	2.2 (0.2)	2.5 (0.0)	3.0 (0.0)	1.5 (0.1)	2.6 (0.3)	3.6 (0.2)
PN mg/L	0.38 (0.04)	0.40 (0.00)	0.37 (0.01)	0.23 (0.03)	0.35 (0.00)	0.35 (0.00)	0.17 (0.01)	0.36 (0.05)	0.43 (0.01)
TSS mg/L	69.2 (26.9)	60.2 (2.8)	78.1 (8.5)	83.2 (78.3)	36.5 (1.5)	78.7 (6.6)	39.5 (4.6)	57.5 (9.3)	121.8 (13.7)
Temp °C	29.5	29.2	29.4 (0.3)	28.3 (0.4)	29.0	27.5	28	28.9	30.2
Chla µg/L	20.9 (1.4)	19.9 (1.4)	26.1 (2.8)	16.5 (1.7)	27.1 (0.9)	46.4 (1.1)	12.5 (0.5)	34.9 (2.4)	34.3 (3.9)

To compare stability of environmental conditions within river sections to between river sections, we determined the coefficient of variation (standard deviation/mean) of each parameter in Table 2.2 for sites within the MMR, within the LMR, and between mean values of the MMR and LMR. The UMR was excluded from this comparison because we sampled only two sites in this river section that were separated by an impoundment and a major tributary. Evident from this comparison is that for most of these parameters there was much less variation within these two river sections than between them (Table 2.3).

Table 3. Comparison of sites for variation in parameter measurements. Values shown are the percentage of the standard deviation/mean (coefficient of variation). For MMR vs LMR, the calculation is based on the means of the two sections. *n* = samples sizes. Parameters and parameter units as in Table 2.2.

Parameter	Within MMR *n = 3 sites*	Within LMR *n = 8 sites*	MMR vs LMR *n = 2 sections*
PO_4-P	7.4	11.9	8.3
NO_3-N	4.1	8.0	45.3
NH_4-N	109.3	94.1	72.4
TDN	5.2	8.7	41.9
TDOC	13.9	21.4	36.5
pH	0.2	1.0	0.9
POC	3.8	9.6	26.2
PN	2.8	13.3	33.0
TSS	10.9	11.6	23.8
Temp	0.9	1.3	2.7
Chla	10.7	10.4	31.9

2.3.2 Sequences

After removal of potentially erroneous, chimeric, and non-bacterial reads, a total of 4,864,418 reads (length = 253 bp) of the V4 region of the 16S rRNA gene were generated. These represented a total of 28,244 bacterial OTUs in the river microbiome, from 106 samples. Particle-associated and free-living bacterial communities were

investigated separately. For sample sites on the Mississippi River, 1,799,962 sequences corresponding to 8,990 OTUs (1,769,980 sequences, 2,202 OTUs following removal of singletons and other rare OTUs) were recovered from free-living samples (n = 37), and 1,934,271 sequences representing 21,149 OTUs (1,861,831 sequences, 4,889 OTUs following removal of rare OTUs) were obtained from particle-associated samples (n = 39). Hence, while the number of reads recovered from the different bacterial samples was similar, there were approximately twice as many OTUs observed in the particle-associated datasets.

An additional 567,710 sequences representing 4,366 OTUs (536,848 sequences, 523 OTUs following removal of rare OTUs) were recovered from free-living samples taken from tributaries (n = 15), while 562,475 sequences representing 10,877 OTUs (490,759 sequences, 958 OTUs following removal of rare OTUs) were obtained from particle-associated samples from tributaries (n = 15).

2.3.3 Downriver Patterns in Bacterial Phyla

We identified 37 phyla of bacteria in the free-living samples taken from the Mississippi River and 49 phyla in the particle-associated fraction. “Dominant” phyla were designated as phyla consisting of >1% of all pooled reads. For both particle-associated and free-living bacteria, the same nine phyla met this criterion (Figure 2.2). Actinobacteria consistently accounted for the highest proportion of sequences in free-living samples along the Mississippi River, while Proteobacteria were dominant in particle-associated samples. Proportions of Bacteroidetes, Cyanobacteria, Planctomycetes, Proteobacteria, and Verrucomicrobia were similar for the two fractions.

Proportions of dominant phyla generally varied only slightly between sites for both free-living and particle-associated communities. The exception was at Memphis, Tennessee, where there was a notable increase in the proportion of Proteobacteria, and a corresponding decrease in the proportion of Actinobacteria among free-living samples, and an increase in the proportion of Firmicutes (and corresponding decrease in the proportion of Cyanobacteria) in the particle-associated fraction. The increase of free-living Proteobacteria was largely attributed to increases of *Acinetobacteria lwoffii* (Figure 2.3A), whereas the increase of particle-associated Firmicutes at Memphis was attributed to increases of *Exiguobacterium* sp. (Figure 2.3B). This shift was transient, and by the following sample site 84 rkm downriver, *A. lwoffii* had declined to pre-Memphis proportions, while among particle-associated phyla the shift also diminished.

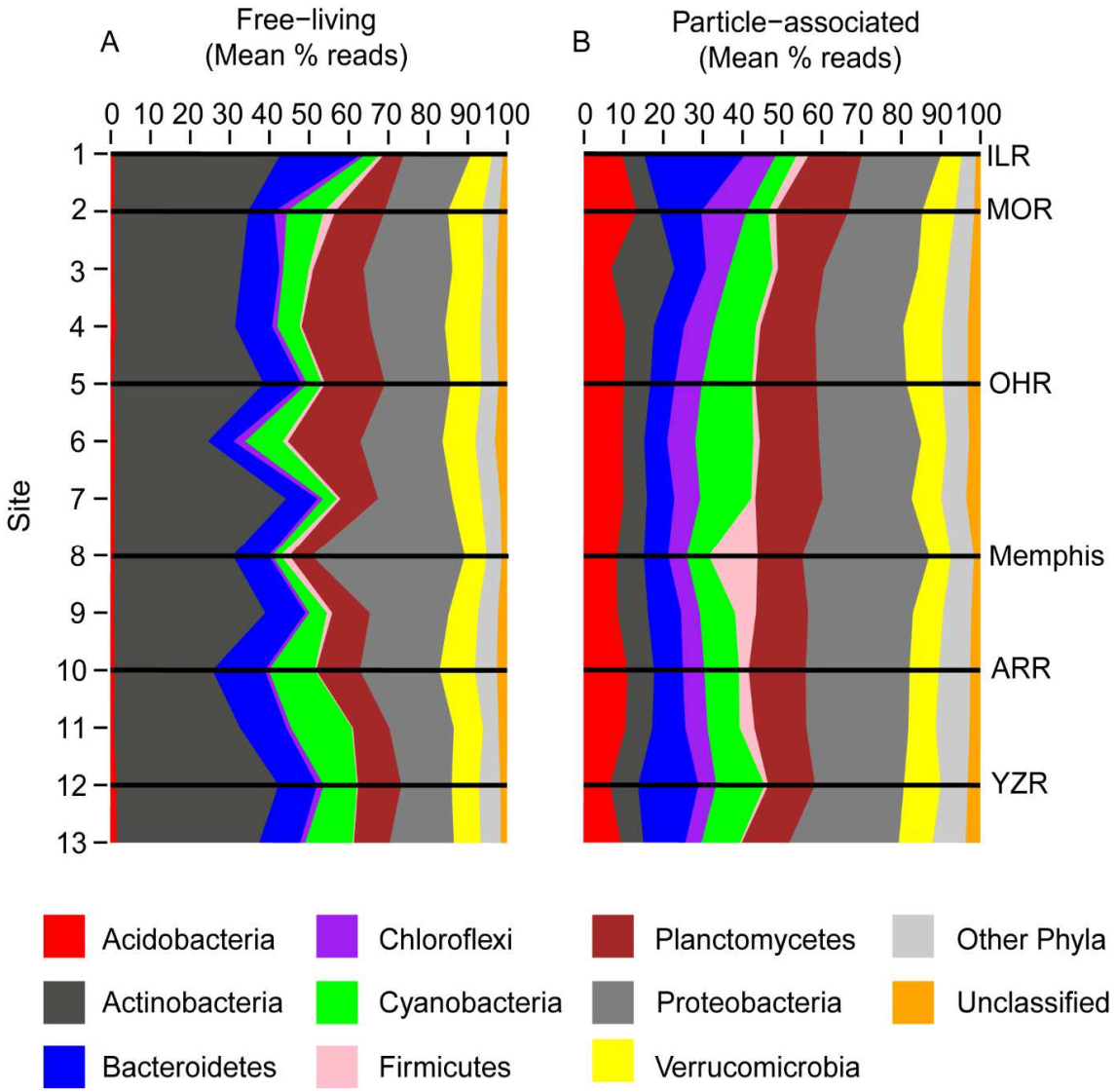

Figure 2. Relative abundances of dominant bacterial phyla sampled along the Mississippi River. Dominant bacterial phyla were defined as comprising >1% of all pooled reads for (A) free-living and (B) particle-associated bacterioplankton communities. Less common phyla (<1% of all pooled reads) are labeled as "Other Phyla", and unclassified bacterial reads are labeled as "Unclassified". Relative abundances are presented as mean percent reads, n = 2-3 per site. Sites on the Mississippi River are numbered sequentially from north to south (Table 1). Black horizontal lines indicate sites on the Mississippi River located just above confluences with tributaries (ILR = Illinois R; MOR = Missouri River; OHR = Ohio River; ARR = Arkansas River; YZR = Yazoo River) and site 8 at Memphis, Tennessee.

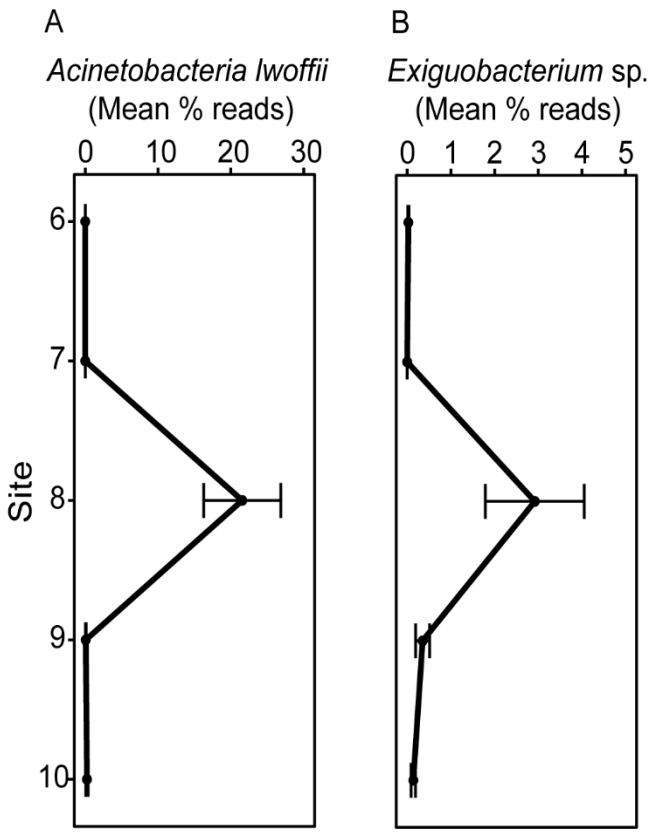

Figure 3. Relative abundances of OTUs identified as (A) *Acinetobacteria lwoffii* and (B) *Exiguobacterium* sp. proximal to Memphis, Tennessee. Relative abundances (mean percent reads ± SE, n = 3 per site) of *Acinetobacteria lwoffii* (free-living bacterioplankton) and *Exiguobacterium* sp. (particle-associated) are presented from sites sampled before, at, and after Memphis (site 8).

2.3.4 Downriver Patterns in Alpha Diversity

Alpha diversity of free-living bacteria, measured as mean OTU richness, varied from 361 ± 63 to 746 ± 31 (mean ± SE) for the Mississippi River sites, with little or no downriver pattern (Figure 2.4A). Particle-associated communities were more diverse, and strongly increased in richness with distance downriver, almost doubling from the north–most (1,007 ± 193 OTUs) to the south-most (1,762 ± 58 OTUs) sample site (Figure 2.4B). Punctuations occurred in the general trend of increasing OTU richness in particle-associated bacteria. Below the confluence with the Missouri River, richness abruptly

increased by 29%, and below its next major tributary, the Ohio River, there was an abrupt decline of 12%.

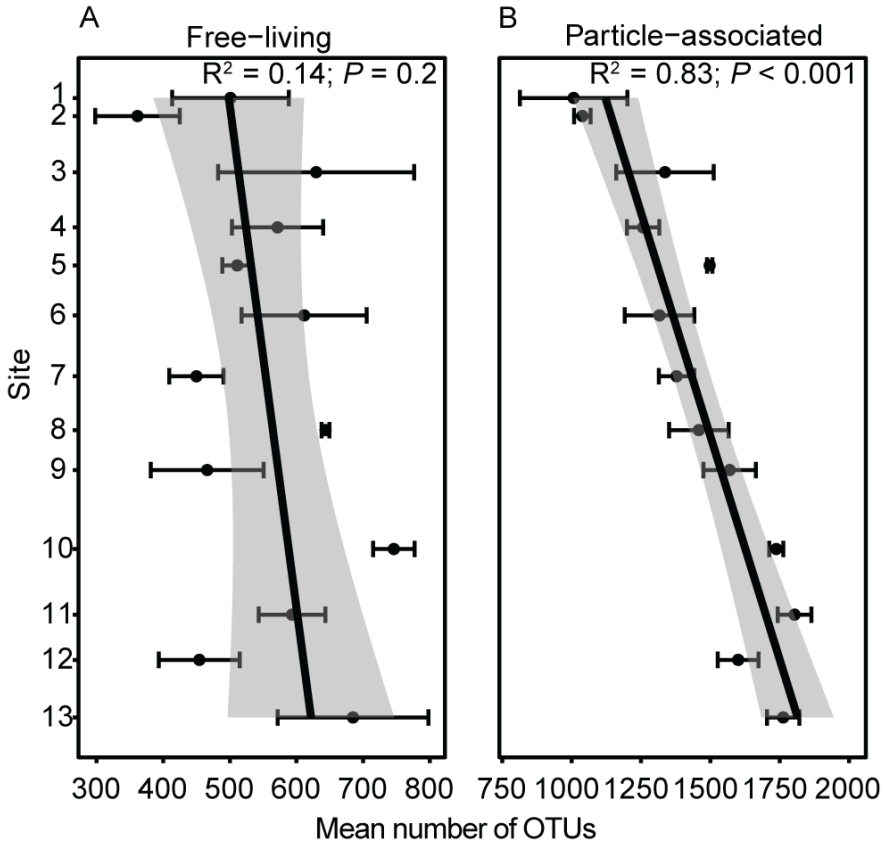

Figure 4. Alpha diversity of (A) free-living and (B) particle-associated bacterioplankton communities sampled along the Mississippi River. Alpha diversity is represented as the number of OTUs (mean ± SE) per sample site (n = 2-3). Sites on the Mississippi River are numbered sequentially from north to south (Table 1). Black lines are fitted regression lines predicting mean OTU number as a function of downriver distance. Gray auras around regression lines represent 95% confidence regions. Statistics for regressions are shown in each panel.

2.3.5 Downriver Patterns in Beta Diversity

Beta diversity, or differences in community composition between sites, was assayed by analyzing the relative abundances of OTUs recovered from each site using the Bray-Curtis dissimilarity index, as visualized by non-metric multidimensional scaling (NMDS) ordinations (Figure 2.5), and comparisons between adjacent sites on the Mississippi River (Table 2.4). These analyses reveal a number of interesting patterns in spatial relationships of microbiome relatedness across this river system. First, the microbiome of each tributary was distinct for both free-living (Figure 2.5A) and particle-associated (Figure 2.5B) bacterial OTUs. Second, both free-living (Figure 2.5A) and particle-associated (Figure 2.5B) components of each tributary microbiome were distinct from the Mississippi River microbiome immediately upstream of the confluence. Third, below each major tributary to the Mississippi (the Illinois, Missouri, and Ohio Rivers) there was pronounced divergence in composition of the post-tributary combined community from the pre-tributary community (Figure 2.5C; Figure 2.5D; Table 2.4). In contrast, within sections of the river between major tributaries, or where the tributary was relatively small compared to the Mississippi River (Arkansas and Yazoo Rivers), there was less variation in the Mississippi River microbiome. There were several exceptions to this general pattern. For the free-living community, below the Ohio River from site 6 to site 7 (Figure 2.5C), there was a large decrease in OTU richness (Figure 2.4A), and consequently a large disjunction in community diversity. For the particle-associated community, the post-Ohio River confluence site (site 6) was similar to the pre-tributary site (site 5) (Figure 2.5D; Table 2.4). Additionally, as noted earlier, at the phylum-level

the microbiome of samples from the Mississippi River near the city of Memphis (site 8), was distinct from other sites in the river (Figure 2.2). These differences were also noted at the OTU-level of analysis, with the Memphis site separating from the other seven sites in the LMR, especially for free-living bacteria (Figure 2.5C). However, with the exception of Memphis, the microbiome from the pre-Memphis site (site 7) to Natchez, Mississippi (site 13), a stretch of 717 rkm without a major tributary, was relatively stable in composition (Figure 2.5, Table 2.4).

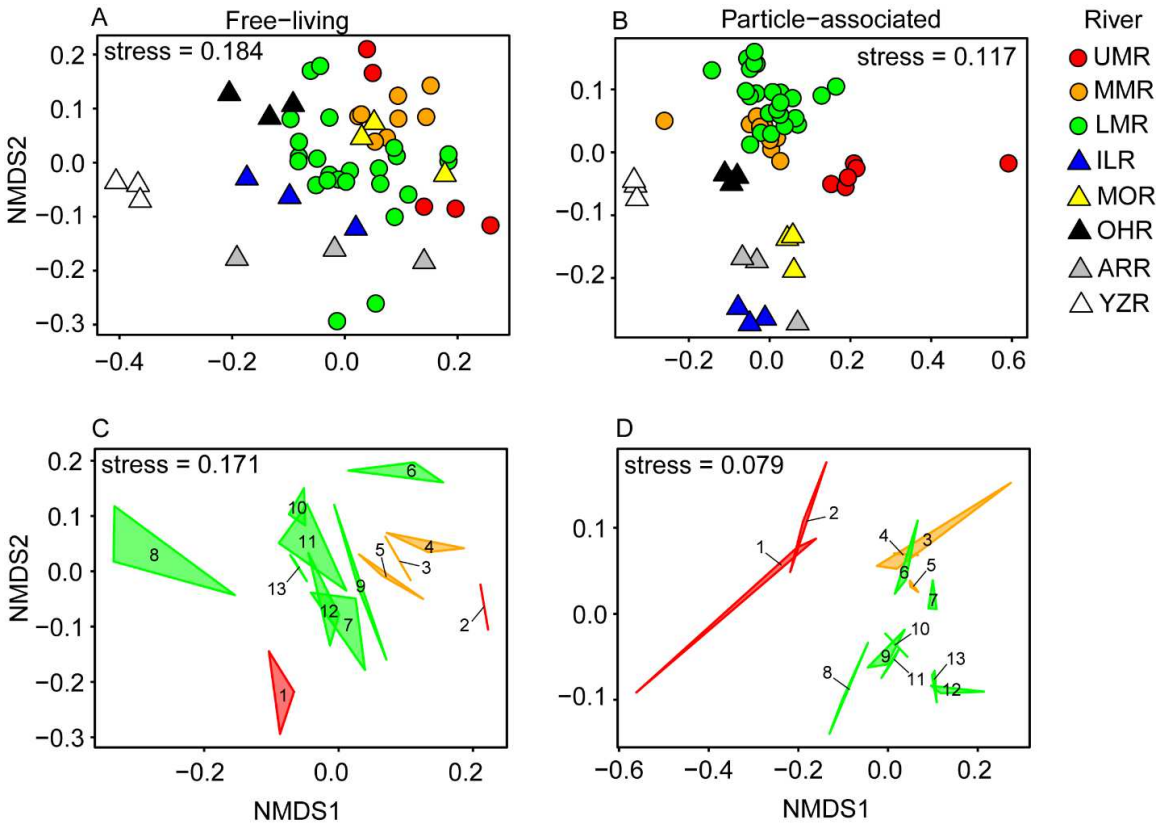

Figure 5. NMDS ordinations of bacterioplankton communities sampled from the Mississippi River system. Ordinations show beta diversity patterns based on Bray-Curtis dissimilarities for (A) free-living and (B) particle-associated communities sampled from the Upper Mississippi River (UMR), Middle Mississippi River (MMR), Lower Mississippi River (LMR), Illinois River (ILR), Missouri River (MOR), Ohio River (OHR), Arkansas River (ARR), and Yazoo River (YZR). Beta diversity patterns for (C) free-living and (D) particle-associated communities from the Mississippi alone are also shown. Images connected at their corners (or line ends) are replicate samples (n = 2-3) from sites on the UMR (red), MMR (orange), and LMR (green). Sites on the Mississippi River are referred to by number, from 1 to 13, from north to south (Table 1). Stress for ordinations are presented in each panel. Ordinations were statistically supported by permutational multivariate analysis of variation (*adonis*; $P < 0.001$ for all).

Table 4. Comparison of Bray-Curtis dissimilarity scores between adjacent sample sites on the Mississippi River. Bray-Curtis dissimilarity scores are presented as mean (standard error). For all comparisons, n = 9 except for sites 2 v 1 (n = 6), sites 3 v 2 (n = 4), and sites 4 v 3 (n = 6). [a] = Site 8 at Memphis was not included because of its apparent influence as a local point source.[b] = Intermediate tributaries are indicated where they occur between adjacent sample sites.

Site comparison[a]	Intermediate tributary[b]	Free-living	Particle-associated
2 v 1	Illinois	0.45 (0.01)	0.49 (0.06)
3 v 2	Missouri	0.39 (0.01)	0.59 (0.03)
4 v 3	-	0.27 (0.02)	0.43 (0.04)
5 v 4	-	0.28 (0.01)	0.33 (0.01)
6 v 5	Ohio	0.39 (0.01)	0.37 (0.01)
7 v 6	-	0.43 (0.01)	0.40 (0.01)
9 v 7	-	0.34 (0.02)	0.45 (0.02)
10 v 9	-	0.37 (0.02)	0.35 (0.01)
11 v 10	Arkansas	0.29 (0.02)	0.30 (0.01)
12 v 11	-	0.33 (0.02)	0.44 (0.02)
13 v 12	Yazoo	0.27 (0.02)	0.37 (0.02)

2.4 Discussion

From examining the bacterioplankton microbiome along a 1,300 rkm downriver transect of the Mississippi River, and concurrently at the mouths of five tributaries to the Mississippi, we can reach several important conclusions about the biogeography of the microbiome(s) of this large river system, and perhaps large rivers in general.

The microbiome of each section of the Mississippi, and each tributary was distinct in composition. For the five tributaries, this result was reported in a prior study of the microbial biogeography of this system conducted during summer 2012 (Jackson et al. 2014). Our results, for a study conducted a year later, confirm that the different rivers of this network have distinct summer microbiomes; additionally, we show this is true for the three major sections of the Mississippi River. As suggested by multivariate correlation analyses of these (Jackson et al. 2014), and other large river networks (Read et al. 2015,

Savio et al. 2015, Staley et al. 2013, 2014, 2015) we assume this spatial pattern in community composition results from differential selection among OTUs in response to the particular environmental properties of each system. An alternative hypothesis to explain inter-river microbiome differences is that exchange of inoculum and colonization among rivers is limited by dispersal barriers. However, considering the various ways in which microbiota might be spread among rivers (wind, barge traffic, animal vectors), even if at low frequency, we think it unlikely that such geographic barriers play an important role compared to the influence of environmental selection (Martiny et al. 2006).

Each river microbiome consisted of free-living and particle-associated assemblages and these two communities were distinct in composition, and presumably biogeochemical processes (Millar et al. 2015). These observations emphasize that even in a turbulent and well-mixed river spatial heterogeneity in microbial community composition is possible at the micro-scale as well as at the macro-scale of river basins (Crump et al. 1999, Jackson et al. 2014, Savio et al. 2015).

Neither the free-living or particle-associated assemblages showed downriver differences in the proportions of dominant bacterial phyla along the Mississippi River, even following major tributary confluences, with the exception of an apparent urban point source of anthropogenic contaminants at Memphis, Tennessee. When sampled over only a two-week period, equilibrium in proportional composition at the phylum-level might be expected for such a broad level of taxonomic resolution, even over so long a river distance. However, it is not at all clear that this would be the case across a longer

span in time, for example over the much greater range in physiochemical conditions corresponding to seasonal transitions.

In contrast to the relatively unvarying downriver patterns in diversity at the phylum-level, there was a clear downriver pattern in alpha diversity measures at the OTU-level. Particle-associated bacteria steadily increased in downriver OTU richness. This pattern, we hypothesize, could be a result of increasing particle heterogeneity or particle colonization, or both, with downriver flow. The relatively large increase in OTU richness below the Missouri River can be explained by the high concentration of suspended sediments entering the Mississippi at this confluence, as the Missouri carries a higher sediment load than the UMR. Conversely, the abrupt decline in richness below the Ohio River may be because of dilution with relatively particle-free Ohio River water (Goolsby et al. 1999, Turner and Rabalais 2004). Community richness of free-living OTUs also appeared to increase with distance downriver but the pattern was not as pronounced. To explain this difference in patterns of downriver richness, a reasonable hypothesis is that habitat heterogeneity in the mixed fluid phase of flow is less than, and does not increase to the same degree as for the suspended particle load.

Beta diversity of the Mississippi River microbiome varied in general agreement with our predictions regarding the spatial pattern in rates of change with downriver transport. Along the Mississippi there was mostly, but not always, gradual variation in microbiome diversity. This pattern of slow change was especially apparent in the 520 rkm stretch south of Memphis, where the bacterioplankton community was relatively stable. In contrast, below confluences with a major tributary (the Illinois, Missouri, and

Ohio Rivers) the microbiome usually, but not always, changed substantially and abruptly in diversity. This was apparent below the Illinois and Missouri Rivers (separating the UMR from the MMR) for both fractions of the microbiome, and for the free-living component below the Ohio River (separating the MMR from the LMR). We assume these downriver punctuations in Mississippi microbiome diversity were due to joining of a major tributary river and its passenger microbiome to the Mississippi microbiome. However, for the Illinois River confluence, there is an alternative explanation. Separating sites 1 and 2 there is the Illinois River, but also a large impoundment, the Melvin Price Dam. This creates more lentic conditions at site 1 and lotic conditions below the dam at site 2, so the change between these two sites in the Mississippi microbiome (Figure 2.5, Table 2.4) may be an impoundment effect, and not only a tributary effect.

One exception to the usual pattern of generally larger changes of microbiome diversity at major river confluences was for particle-associated assemblages in the LMR below the Ohio River confluence compared to the pre-tributary MMR. This exception can be explained by the fact that the Ohio River carries a much less suspended sediment load, and presumably particle-associated microbes, than the MMR (Goolsby et al. 1999, Turner and Rabalais 2004). If this explanation is correct, the particle-associated community below the confluence should be dominated by that of the Mississippi, and this is exactly as we observed. It took 3-4 days, or 325 rkm, of transport below this confluence before the particle-associated component resembled subsequent sites on the LMR. In contrast, from before (site 5) to after (site 6) the Ohio River confluence the free-living microbial community changed relatively strongly, continuing to vary strongly for

another day after that, before relative equilibrium was reached over the last 520 rkm of the sampling reach (sites 9 – 13).

Together, these results suggest a scenario for a pattern in downstream biogeographic variation in river microbiome diversity. At a major confluence, there is neutral mixing of two distinct river microbiomes. This can force a relatively abrupt and large change in diversity of the combined microbiome from the pre-confluence microbiomes, probably by a process of mass immigration. The magnitude of the change will depend on the relative contributions of each river to the merger, a function of individual river discharge and microbiome composition. Following convergence, in response to the new environmental conditions of the merged rivers, and in the absence of other major inputs, there is a more gradual process of microbial community succession. A somewhat similar conclusion for causal factors affecting downriver spatial variation in microbial community structure was reached for the Upper Mississippi River (Staley et al. 2013, 2015).

At Memphis, there was a noticeable change in the microbiome even in the absence of a major tributary input. As opposed to everywhere else, this variation was evident at both low (phylum) and high (OTU) levels of resolution. The bacteria we found associated with the change are indicators of wastewater (Goñi-Urriza et al. 1999, Kulshreshtha et al. 2013), and probably derived from a treatment plant (MC Stiles Plant), that discharges into the Mississippi River north of downtown Memphis. The influence of anthropogenic inputs at this site is confirmed by a recently published finding that in these samples were elevated concentrations of wastewater contaminants including

pharmaceuticals and personal care products (Bussan et al. 2017). Sampling in an area that might be influenced by such a facility was unintentional, but the results are useful for understanding controls on composition of the river microbiome. Although the shift in the microbiome at Memphis was substantial, it was transient and within one day of transport the pre-city river microbiome had become re-established, with relatively little further change with flow over the next four days. These observations suggest that even in a very large river, microbial contaminants in urban wastewater can alter the composition of a native river microbiome, but their survival may be short-lived compared to indigenous taxa, and hence the disturbance only transient in the adapted, or continuously adapting, river microbiome (Staley et al. 2013).

Influenced by its major tributary inputs, the Illinois, Missouri, and Ohio Rivers, the three sections of the Mississippi River (the Upper Mississippi, Middle Mississippi, and Lower Mississippi River) are distinguishable from each other in physical and chemical properties. After one day of flow below confluences with these major tributaries there were usually major shifts in diversity of the Mississippi River microbiome compared to pre-tributary diversity. This is in contrast with stretches of the river more distant in distance and time from major confluences, where changes between adjacent sites in diversity tended to be more gradual. We conclude that while flowing downriver, diversity of the Mississippi River microbiome is modified by point source inputs of external microbiomes from major tributaries draining distinct sub-watersheds, complemented by a gradual process of community succession along intervening stretches of more stable environmental conditions.

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CHAPTER III:

TIMESCALES OF VARIATION IN DIVERSITY AND PRODUCTIVITY OF BACTERIOPLANKTON ASSEMBLAGES IN THE LOWER MISSISSIPPI RIVER

Abstract

Large rivers are characterized by rapid and continuous exchanges in place of flowing habitat and resident plankton. Thus, there is potential for continuous, and possibly abrupt, variation in diversity and metabolic activities of bacterioplankton assemblages on short timescales. However, temporal dynamics of bacterioplankton assemblages of large rivers have seldom been evaluated along a time interval gradient. We quantified variation in alpha and beta diversity and productivity of particle-associated and free-living bacterioplankton assemblages collected at a single site on the Lower Mississippi River at timescales ranging from days to weeks to months up to a year. For both alpha and beta diversity, there were similar patterns of temporal variation in particle-associated and free-living assemblages. Alpha diversity, while always higher on particles, varied at short timescales. In contrast, beta diversity increased with time interval peaking between samples collected 180 days apart. Thereafter, composition became increasingly more similar with sampling interval with gradual reassembly of the microbiome on an annual basis. The primary environmental driver of this temporal pattern was temperature, followed by dissolved nitrogen (N) and chlorophyll *a* concentrations. Beta diversity

patterns were related to changes in the relative abundance of taxa frequently recovered from large rivers. Bacterial productivity was concentrated on suspended particles and corresponded strongly to temperature, while free-living productivity was much lower and constant over time. We conclude that free-living and particle-associated bacterioplankton assemblages in the Lower Mississippi vary in richness, composition, and productivity at distinct timescales in response to differing sets of environmental factors.

3.1 Introduction

In small streams, because of frequent and pronounced environmental disturbances in physical and chemical conditions, variation in microbial assemblage structure may be unrelated to timescale so that assemblages sampled closer in time may be as dissimilar as those sampled months apart (Portillo et al. 2012). In less stochastically disturbed aquatic systems, however, microbial assemblages appear to vary more predictably, and over the same temporal scales in which there is variation in diversity and/or activity of annual plant and animal assemblages (Shade et al. 2013). For example, seasonally recurrent bacterioplankton assemblages have been observed in temperate marine environments (Fuhrman et al. 2006, Gilbert et al. 2012), lakes (Kent et al. 2007, Lindström et al. 2005), and even large rivers (Crump and Hobbie 2005, Fortunato et al. 2012, 2013, Staley et al. 2015) associated with variation in day length, water temperature, hydrology, and nutrient concentrations.

From previous studies of the Mississippi River network, a system of multiple linked large rivers, we observed consistent and pronounced spatial variation in bacterioplankton assemblages. At a microhabitat level, assemblages attached to

suspended particles (i.e. particle-associated bacterioplankton) were richer in bacterial operational taxonomic units (OTUs), and distinct in composition compared to free-living bacterioplankton (Jackson et al. 2014, Payne et al. 2017). At a regional level, assemblages in major tributaries of the Mississippi River—the Illinois, Missouri, and Ohio rivers—were distinct in composition, presumably due to the particular environment of each river (Jackson et al. 2014, Payne et al. 2017). Within the Mississippi River itself, planktonic microbial assemblages flowing downstream exhibited relatively large shifts in diversity after mixing at major confluences, but varied more gradually with increasing distance from confluences (Payne et al. 2017). Clearly, as for other aquatic ecosystems, environmental selection processes structure bacterioplankton assemblages of this river network. But in what taxonomic groups, of what magnitude, over what temporal scales, and in response to exactly what factors do assemblage changes occur? For instance, if one were to sample continuously over time at a single location in a large river, in what respects and in concert with what environmental conditions, would the microbial plankton community vary? These questions address the relative importance to microbial community diversity and activity of stochastic variation over short time periods compared to over longer timeframes, in the context of an ecosystem marked by continuous, directional fluxes of water, chemicals, suspended materials, and microorganisms.

Using Illumina sequencing of the bacterial 16S rRNA gene, we measured variation in alpha diversity (within-sample richness of OTUs) and beta diversity (between-sample differences in composition) within and between particle-associated and

free-living bacterioplankton assemblages at a single site in the main channel of the Lower Mississippi River over a range of temporal scales. Assemblages were collected on a daily and weekly basis in summer, and monthly over a year. Additionally, on each sampling date, we measured bacterial productivity and habitat variables. From these measurements, we determined the relationship of timescale to variation in bacterial diversity and productivity, and identified likely environmental drivers of variation. We hypothesized that diversity and productivity would scale with time in relationship to environmental change, and therefore be less variable at shorter than over longer timescales.

3.2 Methods

3.2.1 Sample site and water collection

The Lower Mississippi River was sampled on 23 dates between February 2013 and January 2014 (Figure 3.1A), directly off Mhoon Landing (34°10'37.59" N, 90°27'06.64" W), near Tunica, Mississippi (Figure 3.1B). Mhoon Landing is 76 river kilometers (rkm) below Memphis, Tennessee, and 426 rkm below Cairo, Illinois, where the Ohio River joins the Mississippi River, forming the Lower Mississippi. At Mhoon Landing the river is turbulent with little evidence of vertical stratification in dissolved chemistry (Ochs et al. 2010). Discharge ranges from roughly 7,000 to 27,000 $m^3 s^{-1}$ (Baker et al. 1991) or occasionally higher, depending on time of year (Figure 3.1A).

Sampling spanned three temporal scales (Figure 3.1A). Samples were collected once monthly, near the beginning of each calendar month, from 2 February 2013 to 11 January 2014, for a total of 12 monthly samples. At a finer scale, samples were collected weekly from 3 June to 15 July 2013, for a total of seven weekly samples. Finally,

samples were collected daily from 24 June to 1 July 2013, for a total of eight daily samples. On each date, sampling occurred between 10:00 and 13:00 h, and water was collected from mid-river at a depth of 0.5 m. Sterilized 1-L Nalgene sample bottles (n = 3) were used to collect water for chemical analyses and heterotrophic bacterial productivity, and sterilized 500-mL Nalgene sample bottles (n = 3) were used to collect water to analyze bacterioplankton assemblage structure. All bottles were stored in coolers containing river water to maintain ambient temperature during transportation (0.5-1.5 h) to the laboratory for additional measurements, sample fractionation, and preservation.

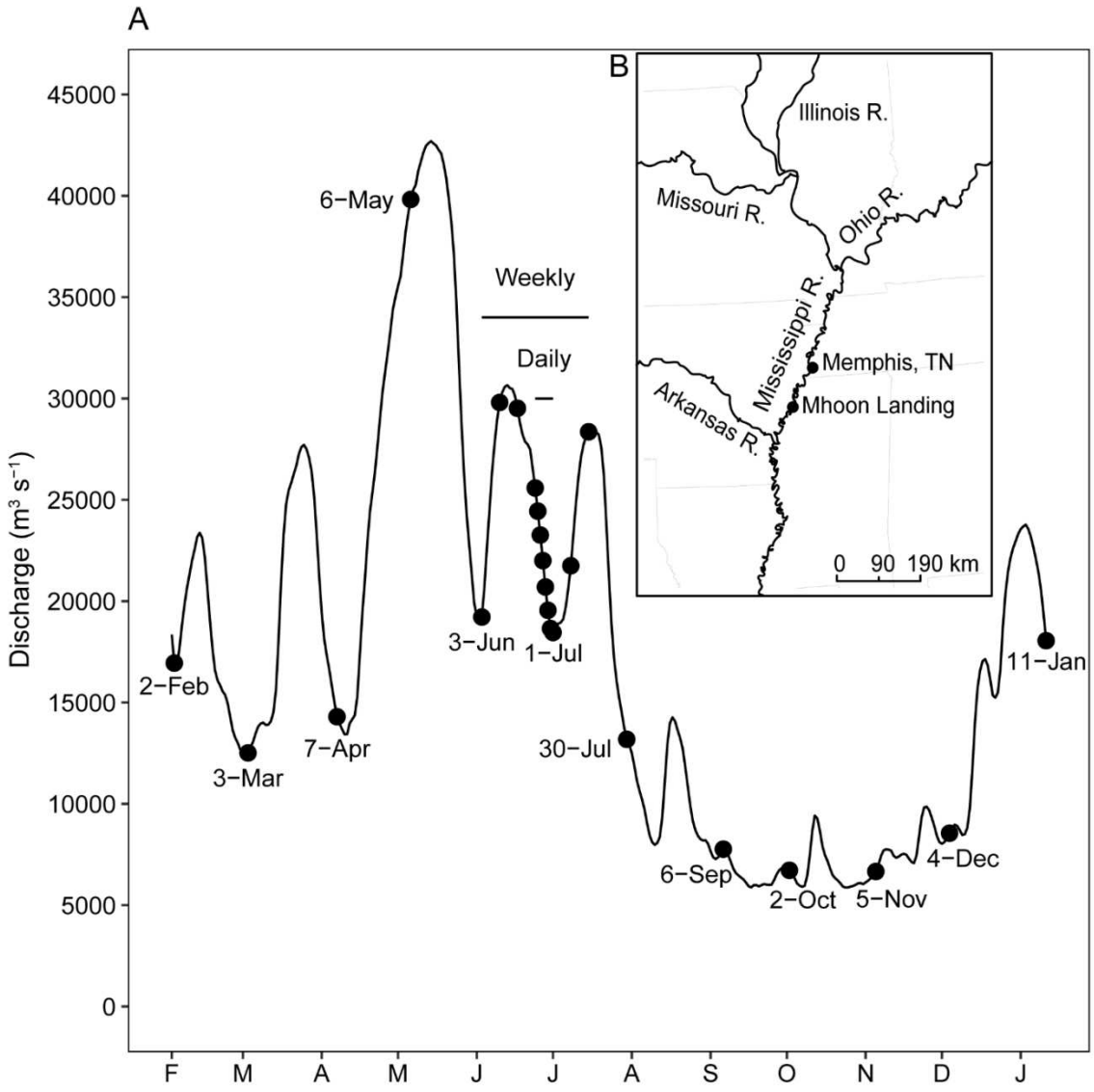

Figure 6. (A) Hydrograph of discharge of the Lower Mississippi River at Mhoon Landing, Mississippi between February 2013 and January 2014. Points on hydrograph represent sample dates. Monthly sample dates (n = 12) are labeled by date, while horizontal bars indicate weekly (3 June to 15 July 2013, n = 7) and daily sampling (24 June to 1 July 2013, n = 8) periods. Discharge measurements were calculated using gage height data collected daily by the U.S. Army Corps of Engineers at Helena, Arkansas located 40 rkm below Mhoon Landing. (B) Map of a portion of the Mississippi River Basin indicating sample location (Mhoon Landing) relative to Memphis, Tennessee, and major river tributaries.

3.2.2 Environmental measurements

Water temperature was measured in the field using a Hawkeye Digital Sonar H22PX-B. In the laboratory, sub-samples (100-200 mL) were filtered through ashed 47-mm diameter, Whatman GF/F filters. For preservation, filters and filtrates were frozen at -60 °C or -20 °C, respectively. Total suspended sediment (TSS) concentrations were measured gravimetrically on filters after drying at 60 °C. Chlorophyll *a* (Chla) concentrations were assayed by spectrophotometry of pigments extracted in 90% NH_4OH-buffered acetone for 24 h at 5 °C (Wetzel and Likens 2000). Total dissolved organic C and total dissolved N were measured in filtrates using a Shimadzu Total Organic Carbon Autoanalyzer, while total dissolved P concentrations were assessed using standard spectrophotometric methods (Wetzel and Likens 2000). C:N, N:P, and C:P molar ratios were calculated from these measurements.

3.2.3 DNA extraction and sequencing

From the 500-mL sample bottles, 100-mL subsamples were removed for serial filtration (<5 mm Hg vacuum). Subsamples were initially passed through sterile Millipore 3-µm pore-size polycarbonate filters, and the filtrate immediately filtered through sterile Millipore 0.22-µm pore-size polyethersulfone filters. Particles collected in the first filtration include particle-associated cells or cells >3 µm in size. Particles collected in the second filtration step include smaller (0.22-3-µm) bacteria, assumed to be mostly free-living (Jackson et al. 2014, Payne et al. 2017). Filters were stored at -20 °C before molecular processing.

DNA was extracted from filters using PowerWater DNA isolation kits (MoBio, Carlsbad, California). The bacterial 16S rRNA gene was amplified and sequenced using methods modified from Kozich et al. (2013), and described previously (Stone and Jackson 2016, Payne et al. 2017). Briefly, the V4 region of the bacterial 16S rRNA gene was amplified using standard forward (5'-GTGCCAGCMGCCGCGGTAA) and reverse (5'-GGACTACHVGGGTWTCTAAT) primers adapted with dual-index barcodes for Illumina MiSeq next generation sequencing (Kozich et al. 2013), and run through 30 cycles of denaturation (95 °C) for 20 s, annealing (55 °C) for 15 s, and elongation (72 °C) for 2 min, and a final elongation (72 °C) for 10 min. PCR products were normalized by sample using SequalPrep Normalization Plates (Life Technologies, Grand Island, New York), pooled, and sequenced using an Illumina MiSeq platform located at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center. All sequences can be accessed in the NCBI SRA database under the BioProject ID PRJNA358603.

3.2.4 Sequence processing and analysis

Sequence data were processed using the bioinformatics software *mothur* (Schloss et al. 2009) by a procedure modified from Payne et al. (2017). Briefly, the SILVA rRNA database (release 119) was used to align sequences with reference V4 sequences (Pruesse et al. 2007), and all unaligned sequences were discarded in addition to homopolymers >8 bp. Before classification, sequences differentiated by ≤2 bp were merged, and potential chimeras identified by UCHIME (Edgar et al. 2011) removed. Sequences were classified using the RDP database (Release 11, September 2016) (Cole et al. 2014). Non-bacterial

lineages (e.g. Archaea, Eukarya, and mitochondria) were then removed. As RDP classification does not distinguish between cyanobacteria and chloroplast lineages at the phylum-level, chloroplast sequences were removed in a subsequent step (see below). Finally, all remaining sequences were clustered into OTUs based on ≥97% similarity.

Sequence data were processed further and analyzed in R version 3.5.1 (R Core Team 2013). OTU and taxonomy tables generated by *mothur* were imported into R and merged with environmental metadata using the microbiome analysis software *phyloseq* version 1.14.0 (McMurdie and Holmes 2013). OTUs identified as belonging to chloroplast lineages were removed from the dataset. Seven free-living bacterial samples (single replicates from 2 February, 4 April, 1 July, 8 July, and 15 July, and two replicates from 3 June) had poor sequencing depth (<3,500 sequences per sample) (Lundin et al. 2012) and were removed. The remaining 3 June free-living sample was also removed so that each date was represented by at least two replicates. Univariate statistics were performed using the R package *car* (Fox and Weisberg 2011), while multivariate statistics were performed using either *phyloseq* or *vegan* version 2.5-3 (Oksanen et al. 2018). Graphics were generated using the R package *ggplot2* version 2.1.0 (Wickham 2016).

Alpha diversity (i.e. richness of bacterial OTUs) within samples was determined from an untrimmed dataset (i.e. containing singleton OTUs) using the phyloseq function "estimate_richness". Levene's Test was used to detect homogeneity of variance in bacterial alpha diversity between particle-associated and free-living samples, and

between samples collected weekly and daily from 3 June to 15 July versus those collected over longer timescales (i.e. from 2 February to 11 January, excluding summer samples).

Beta diversity (i.e. differences in composition) between bacterial samples was evaluated after removal of singleton OTUs and standardization of sequence libraries to 3,629 sequences each (i.e. smallest sequence depth). Beta diversity was quantified using Bray-Curtis dissimilarity matrices. To visualize whether bacterial samples collected closer in time were more similar in composition, mean pairwise dissimilarities were plotted against Euclidian distances in sample date. Differences in composition between particle-associated and free-living samples were also visualized using non-metric multidimensional scaling (NMDS) ordinations. Envfit (package *vegan*) analysis was then used to determine abundant bacterial OTUs that correlated with separation of samples in NMDS space.

Permutational multivariate analysis of variance (function "adonis" in the package *vegan*) was used to test for significant differences in beta diversity between groups of samples (e.g. between particle-associated and free-living, or between samples collected at short and long timescales) (Anderson 2001). Permutated distance-based test for homogeneity of multivariate dispersion (function "PERMDISP2" in the package *vegan*) was then used to test for significant differences in the variance in beta diversity between sample groupings (Anderson 2006).

Environmental drivers of particle-associated and free-living beta diversity were determined by distance-based redundancy analysis (dbRDA) generated using function "capscale" in the package *vegan*. Before dbRDA was run, stepwise forward selection

modeling (function "ordiR2step" in the package *vegan*) was used to find the best combination of variables that explained variation in composition (Blanchet et al. 2008). Environmental measurements in initial models were: temperature, C:N, N:P, C:P, TSS, and Chla. After forward selection modeling, a variance inflation test (function "vif.cca" in the package *vegan*) was used to identify redundant constraints. If multiple variables had a variance inflation factor greater than 10, the variable with the lowest value was removed from the model, and forward selection was rerun. Models were then tested for significance using analysis of variance (ANOVA). Variables that were both selected by "ordiR2step" and had marginal significance were analyzed using dbRDA.

Standardized samples were also used to assess patterns of variation in relative abundances of bacterial OTUs. Plots were created in package *ggplot2* using the function stat_smooth. Local polynomial regression fitting (function "loess" in package *ggplot2*) was used to display patterns of variation in relative abundances. 95% confidence intervals were plotted around regression lines.

3.4.5 Bacterial productivity measurements

Bacterial productivity was determined based on radio-labeled isotope incorporation. Leucine (^{3}H-leucine) and thymidine (^{3}H-thymidine) (Moravek Biochemicals) at specific activities of approximately 100 Ci mmol^{-1} were used to determine synthesis rates of proteins and DNA, respectively (Wetzel and Likens 2000). Productivity of the total assemblage was measured using whole-water samples, while productivity of free-living cells only was measured in sample water filtered through sterile 47-mm diameter, 3-µm Millipore polycarbonate filters (Ochs et al. 2010).

Productivity measurements were made using a microcentrifuge procedure modified from Kirchman (2001). Triplicate bulk and filtered water samples (1.5 mL) were added to 2-mL microcentrifuge vials along with a saturating concentration of 60 nM ^{3}H-leucine or ^{3}H-thymidine (Ochs et al. 2010). A control tube for every treatment was prepared by adding trichoroacetic acid (TCA) immediately after isotope addition (see below). Thus, there were a total of 16 vials used per sample period. Incubations were generally initiated in the field beginning immediately after sample collection. Vials were incubated in river water at ambient temperature for 1 h, then placed on ice for 5 min, after which 94 µL of 80% TCA was added to halt isotope uptake. In the laboratory, vials were centrifuged at 18,000 rpm for 10 min, and the supernatant removed. Cold 5% TCA (1 mL) was then added to each vial followed by vortexing, centrifugation, and removal of supernatant. Finally, 1 mL of ice-cold 80% ethanol was added, followed by the washing steps above. Pellets were dried at room temperature overnight, and 1 mL of Fisher ScintiSafe Plus 50% scintillation fluid added to vials, followed by further vortexing. Radioassays were run on a Perkin-Elmer Tri-Carb 2810 TR liquid scintillation counter. Radioisotope-uptake calculations for ^{3}H-leucine representing biomass production, and ^{3}H-thymidine representing cell reproduction, were made as explained in Wetzel and Likens (2000). Productivity of all cells (whole-water) and free-living cells (<3-µm fraction) was determined directly, while productivity of particle-associated (or larger than 3 µm) cells was determined by difference.

3.3 Results

3.3.1 Patterns in the river environment

Over the course of the study, water temperature ranged from <5 °C in winter to 30 °C in summer (Figure 3.2). Dissolved C:N and N:P corresponded closely (inversely and directly, respectively) to the pattern in the river hydrograph (Figure 3.1A). Dissolved C:P, in contrast, did not vary with discharge. TSS concentrations peaked during high discharge in spring or summer, while Chla concentrations were at a maximum during low discharge in autumn. Seasonal and annual variability of these variables in the Lower Mississippi River are tightly coupled with climatic and hydrologic conditions inherent to the river's large watershed, as documented previously (Goolsby et al. 1999, Turner and Rabalais 2004, Ochs et al. 2010).

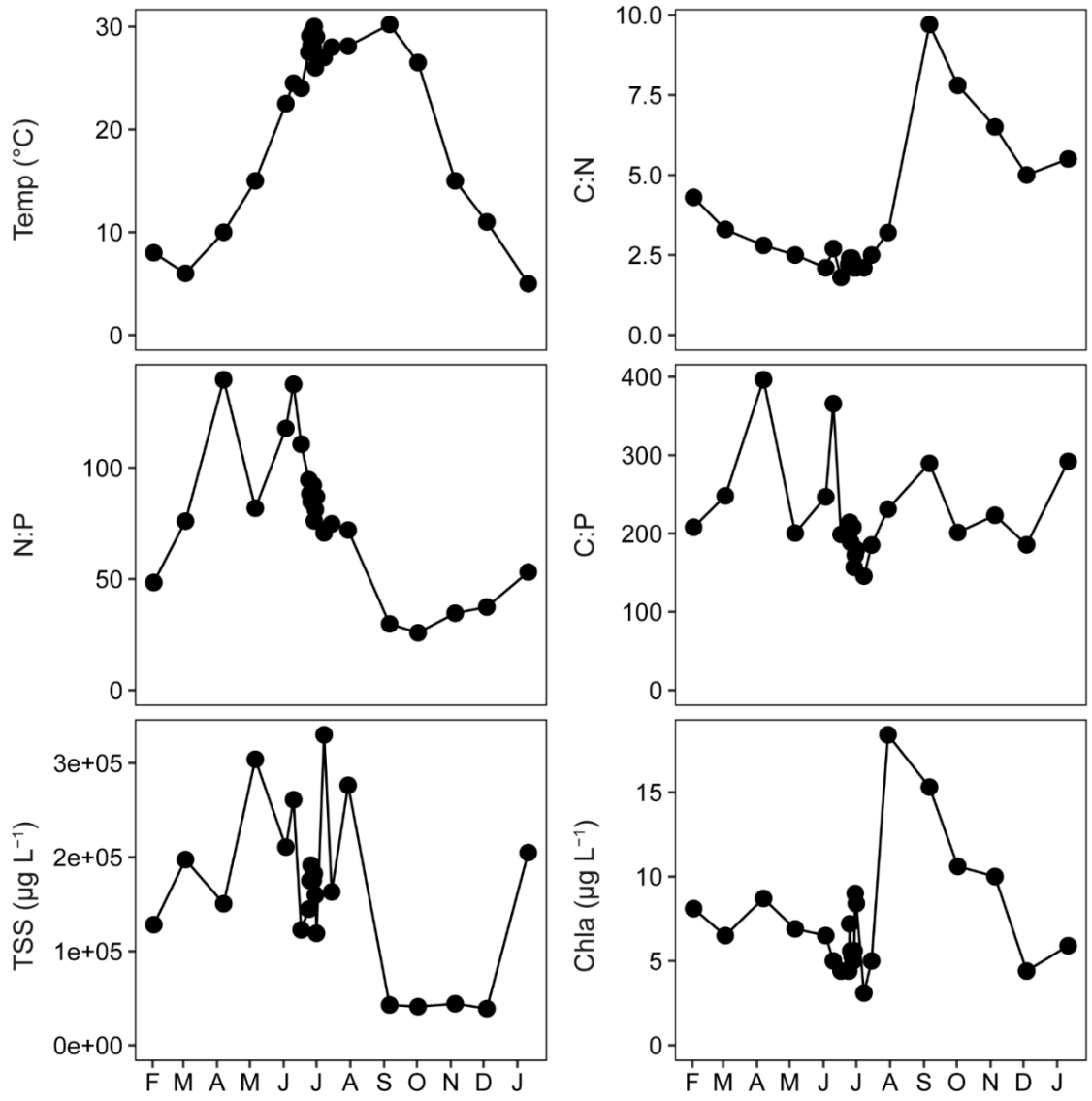

Figure 7. Environmental variables measured in Lower Mississippi River water between February 2013 and January 2014. Abbreviations: Temp, water temperature; TSS, total suspended solids; Chla, chlorophyll a. Except for water temperature, parameter measurements are presented as means for each date, n = 2-3. For clarity, sample dates are connected by lines. These lines are not intended to convey patterns of variation at shorter time intervals than what is shown.

To compare patterns in the timescales of variation, for each environmental variable we calculated the coefficient of variation (CV) for measurements taken daily and weekly (3-Jun – 15-Jul) at short timescales (n = 13), versus those taken on a monthly basis (2-Feb – 11-Jan) at longer sampling intervals (n = 10). For nearly all variables, relative variation increased with timescale of measurement (Table 3.1).

Table 5. Coefficient of variation (%) in environmental variables at short (daily and weekly) and long (monthly) timescales. Abbreviations: Temp, water temperature; TSS, total suspended solids; Chla, chlorophyll *a*. n represents the number of dates per sampling interval.

Variable	Short (n = 13)	Long (n = 10)
Temp	8	61
C:N	10	47
N:P	20	57
C:P	27	26
TSS	31	71
Chla	29	46

3.3.2 Patterns in bacterial alpha diversity

We observed that bacterial alpha diversity (i.e. richness of OTUs) was greater within particle-associated components compared to the free-living counterpart on all dates (range = 1.1 to 7.1 times) (Figure 3.3A). Over the year, the degree of variability in richness was not significantly different between the different components of the microbial community (Levene's Test, $p = 0.264$), and richness peaked within assemblages collected in mid-summer (Figure 3.3A). The variance in particle-attached richness was not significantly different (Levene's Test, $p = 0.398$) between short and long timescales (Figure 3.3B), while there was a greater degree of variability (Levene's Test, $p = 0.043$) in free-living richness at short time intervals (Figure 3.3C).

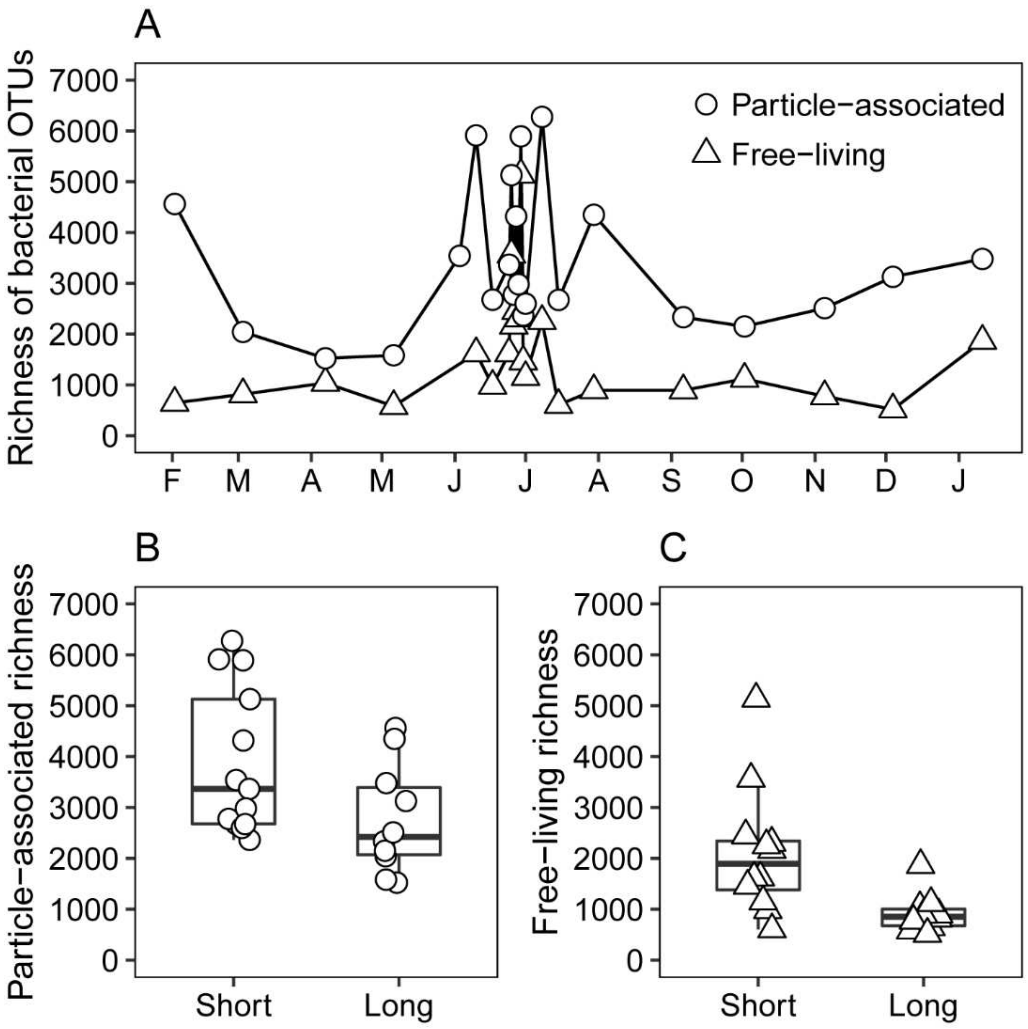

Figure 8. Temporal patterns in bacterioplankton alpha diversity measured using richness of OTUs. (A) Differences in richness of OTUs in particle-associated and free-living bacterioplankton assemblages collected on 23 dates from February 2013 to January 2014. The richness of OTUs are presented as means for each date, n = 2-3. Boxplots compare variance in (B) particle-associated and (C) free-living richness between short and long timescales. Symbols indicate samples.

3.3.3 Patterns in bacterial beta diversity

In general, both particle-associated and free-living components were less dissimilar (i.e. more similar) in composition on daily and weekly timeframes than on a monthly timeframe. However, the pattern was not linear for either group. Instead, dissimilarity exhibited a roughly parabolic pattern (Figure 3.4A). Assemblages became increasingly dissimilar in composition with separation in time up to six months, after which the trend was for a gradual increase in similarity. If we disregard year, these trends indicate that assemblages occurring closer in time, whatever the time of year, are increasingly alike in composition. Furthermore, this pattern of nonlinearity suggests that the Lower Mississippi microbiome varies along seasonal gradients, reassembling on an annual basis.

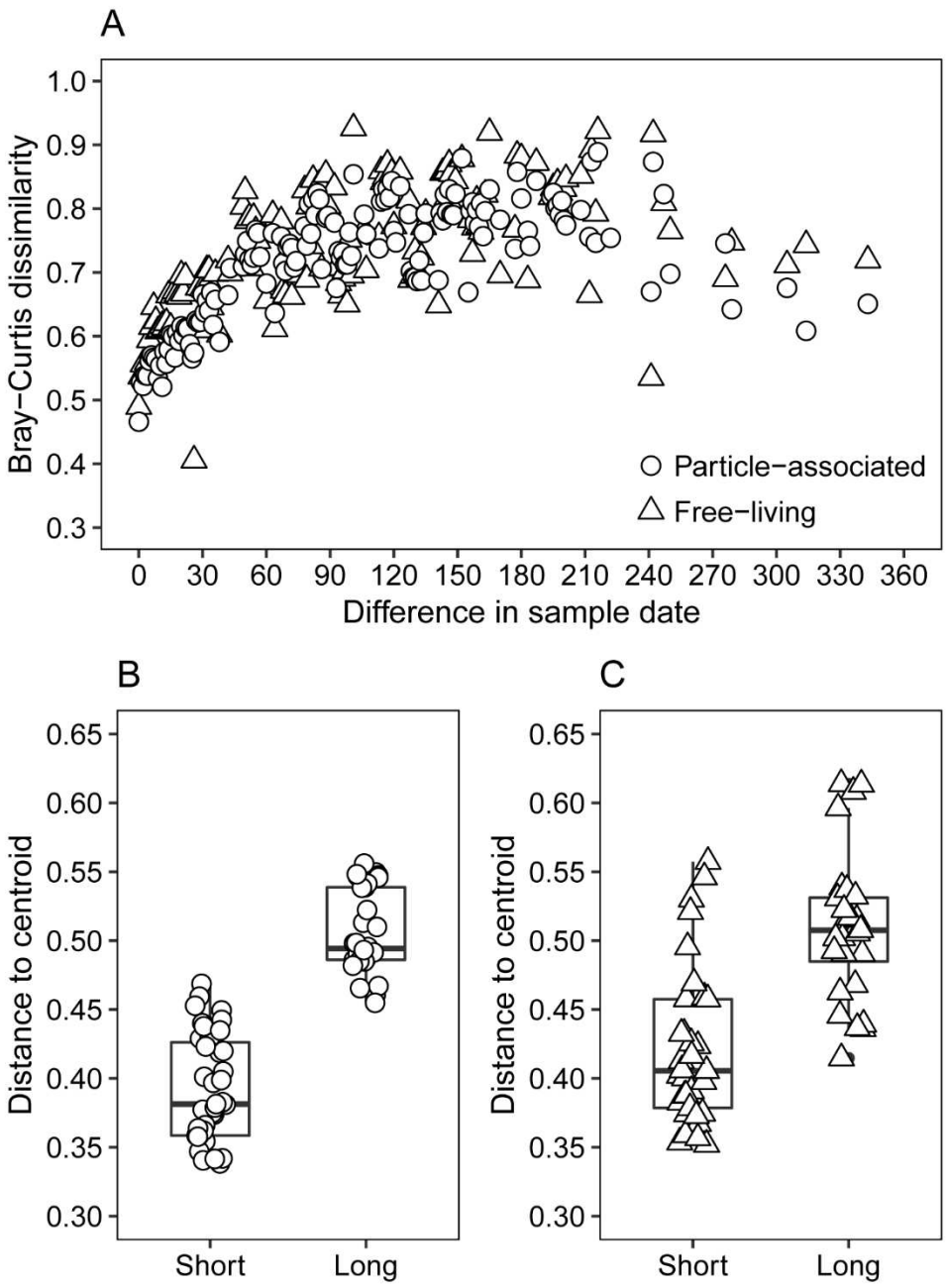

Figure 9. Temporal patterns in bacterioplankton beta diversity measured using Bray-Curtis dissimilarity. (A) Relationships between particle-associated and free-living dissimilarities and interval of time between sample dates. Points represent mean pairwise dissimilarities calculated from bacterioplankton assemblages collected between 1 and 343 days apart. Boxplots compare variance in (B) particle-associated and (C) free-living beta-diversity between short and long timescales. Symbols indicate samples.

While particle-associated and free-living assemblages were distinct in composition (adonis: $R^2 = 0.08$, $p < 0.001$), they were similar in their degrees of variability in composition (PERMDISP2, $p = 0.172$). Furthermore, there was significantly less variability ($p < 0.001$) in beta diversity at short timescales than at long timescales for both particle-associated (Figure 3.4B) and free-living (Figure 3.4C) components.

Variables used in dbRDA explained 33% and 31% of the variation in particle-associated and free-living assemblage composition, respectively (Table 3.2). Variation in microbiome beta diversity was associated primarily with water temperature, and this parameter explained 20% and 19% of the variation in particle-associated and free-living assemblages, respectively (Table 3.2). Variation in composition was explained secondarily by factors closely associated with the river's hydrograph, with C:N explaining 8% of the change in the particle-associated component and Chla explaining 8% of the variation in free-living assemblages.

Table 6. Environmental and biological variables selected by stepwise forward modeling (ordiR2step) that best explained variation (adjusted R^2) in particle-associated and free-living assemblage composition Abbreviations: Temp, water temperature; TSS, total suspended solids; Chla, chlorophyll *a*.

Assemblage	Model	Adjusted R^2
Particle-associated	All variables	0.33
	Temp	0.20
	C:N	0.08
	N:P	0.02
	Chla	0.02
	TSS	0.01
Free-living	All variables	0.31
	Temp	0.19
	Chla	0.08
	N:P	0.03
	TSS	0.01

3.3.4 Patterns in relative abundances of bacterial taxa

At a broad taxonomic level, particular bacterial phyla exhibited distinct patterns in their proportional abundance over the year (Figure 3.5). Proportions of Proteobacteria were fairly constant over much of the sampling period, but trended upward from November to January in both particle-associated and free-living components. Relative abundances of other phyla, in contrast, were more closely related to seasonal changes in water temperature and/or the river hydrograph. Sequences classified as Bacteroidetes and Verrucomicrobia were abundant in assemblages collected in cooler water in spring and winter. Decreased proportions of these taxa, in particular Bacteroidetes, in warm river conditions corresponded with increased proportions of Acidobacteria in summer, and Planctomycetes throughout summer and fall, suggesting a positive influence of increasing temperature on those taxa. Cyanobacteria increased in proportion in late-summer and into early fall when the river was at a minimum in discharge, TSS load, and turbidity.

Proportions of Actinobacteria increased from late-summer to winter, after which they strongly dominated free-living assemblages during the period of least discharge from mid-July to December. However, members of this phylum were much less abundant in particle-associated assemblages sampled during this time.

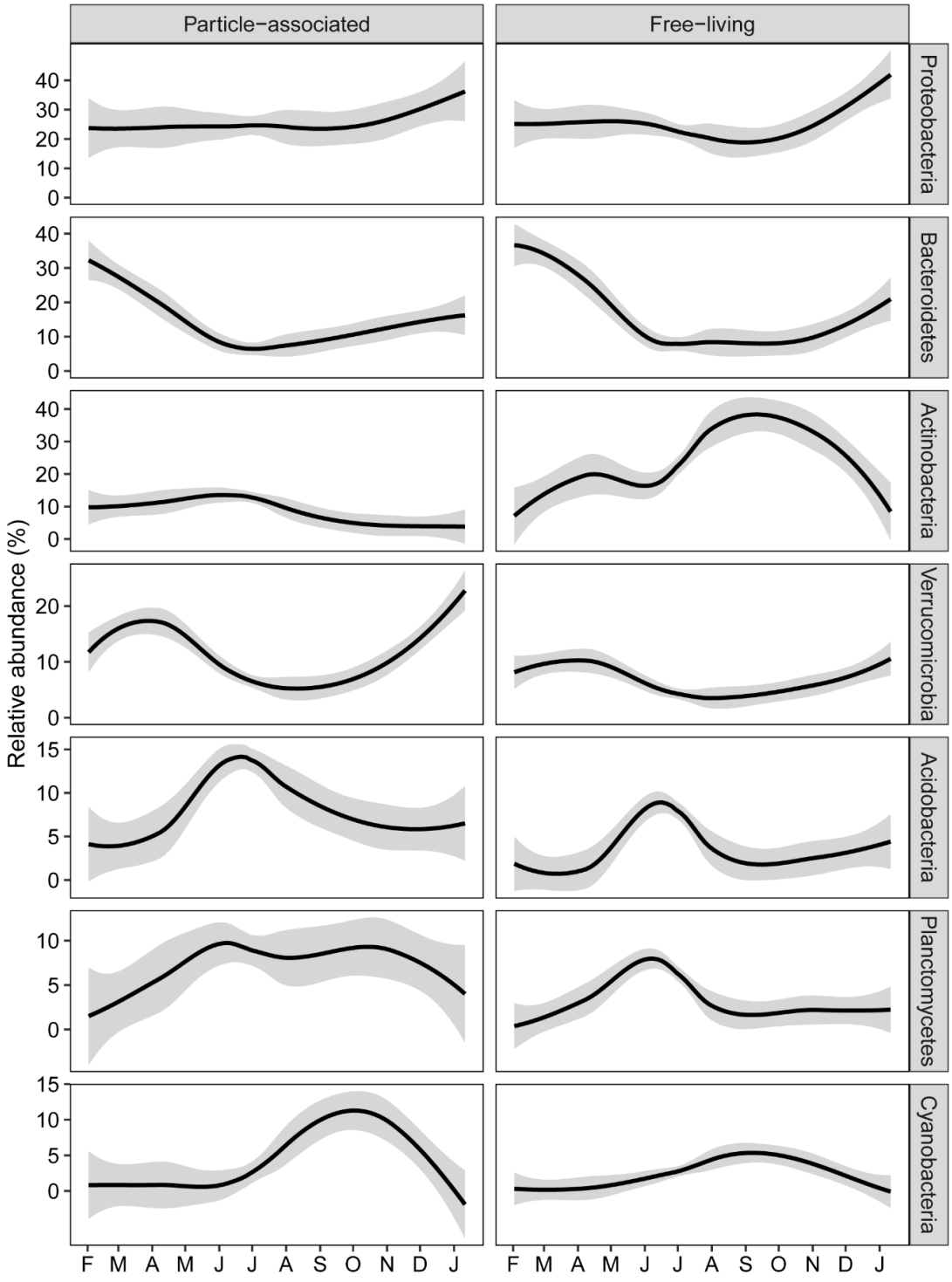

Figure 10. Temporal patterns in relative abundances of bacterial phyla sequenced from particle-associated and free-living bacterioplankton assemblages. Lines were made using local polynomial regression fitting (l*oess*). Shading around lines indicate 95% confidence intervals.

A NMDS ordination confirmed these seasonal patterns of change in composition of particle-associated and free-living bacterioplankton assemblages (Figure 3.6). Particle-associated assemblages separated in time in a clockwise pattern in NMDS space, revealing changes in composition over time in a gradual and predictable manner. While the cyclical pattern was not apparent for the free-living fraction, the ordination shows that both particle-associated and free-living assemblages collected nearly a year apart trended towards similarity in composition.

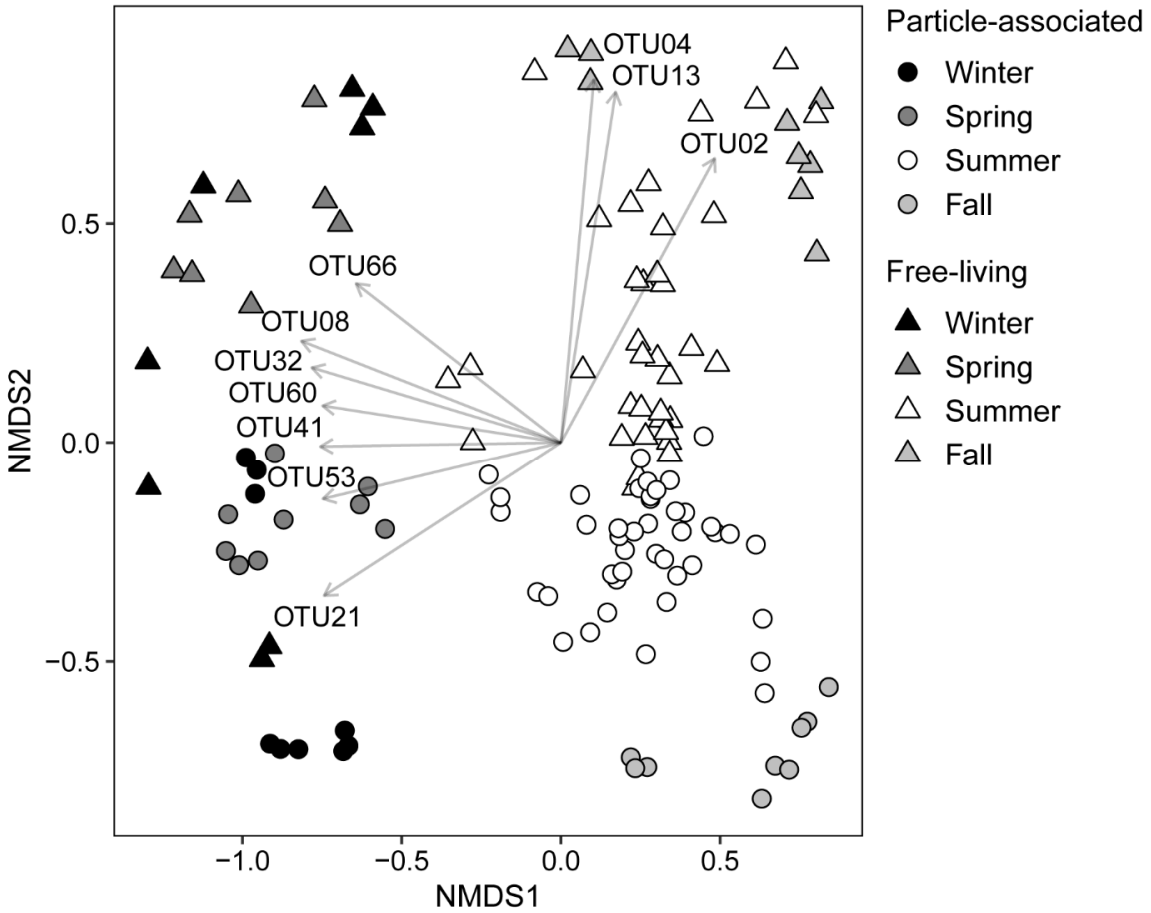

Figure 11. A NMDS ordination showing seasonal changes in composition of particle-associated and free-living bacterioplankton assemblages. Stress for the ordination equaled 0.11. Arrows indicate bacterial OTUs correlated (Envfit analysis: R2 = 0.55 – 0.72, p = 0.001) with the ordination. Identifications of OTUs (RDP classification) are as follows: (OTU02 and OTU04) order Actinomycetales (Actinobacteria); (OTU08) family Comamonadaceae (Betaproteobacteria); (OTU13) class Betaproteobacteria (Proteobacteria); (OTU21) Prosthecobacter (Verrucomicrobia); (OTU32) Methylophilus (Proteobacteria); (OTU41 and OTU53) Flavobacterium (Bacteroidetes); (OTU060) phylum Bacteroidetes; and (OTU66) family Cytophagaceae (Bacteroidetes). Complete identifications of OTUs and specific R2 values of correlations are presented in Table 3.3.

Envfit analysis identified several OTUs that were correlated ($R^2 \geq 0.55$) with bacterioplankton assemblages collected in spring and winter (Figure 3.6; Table 3.3). These OTUs were members of Bacteroidetes (OTU41, OTU53, OTU60, and OTU66), Betaproteobacteria (OTU08 and OTU32), and Verrucomicrobia (OTU21). The associations between bacterial OTUs and assemblages collected in summer were weaker in comparison; however, free-living assemblages in late-summer and fall correlated with OTUs identified to the Actinobacteria order Actinomycetales (OTU02 and OTU04) and an unclassified member of Betaproteobacteria (OTU13).

Table 7. OTUs that correlated (Envfit analysis, R2) with bacterioplankton assemblages plotted in NMDS space. OTUs were classified using the RDP database (release 11, September 2016).

OTU	Phylum	Class	Order	Family	Genus	R^2
OTU02	Actinobacteria	Actinobacteria	Actinomycetales			0.65
OTU04	Actinobacteria	Actinobacteria	Actinomycetales			0.70
OTU08	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		0.72
OTU13	Proteobacteria	Betaproteobacteria				0.67
OTU21	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Prosthecobacter*	0.68
OTU32	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	*Methylophilus*	0.64
OTU41	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.57
OTU53	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	0.58
OTU60	Bacteroidetes					0.57
OTU66	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae		0.55

3.3.5 Patterns in bacterial productivity

Rates of whole-water bacterial productivity measured by the two radioisotopes were similar, ranging from about 30 to 300 nmol C L^{-1} h^{-1} (Figure 7A, B). The temporal pattern correlated strongly with temperature, $R^2 = 0.68$ and 0.78 for ^{3}H-leucine and ^{3}H-thymidine incorporation, respectively ($p < 0.001$ for each), increasing from spring through late summer, and declining to minimum values in winter. Particle-associated productivity was usually much greater than for free-living cells. On average, attached bacteria represented 87.9% (standard error = 2.3%) of new biomass measured by protein synthesis, and 89.3% (standard error = 2.7%) measured by rates of cell division in whole-water.

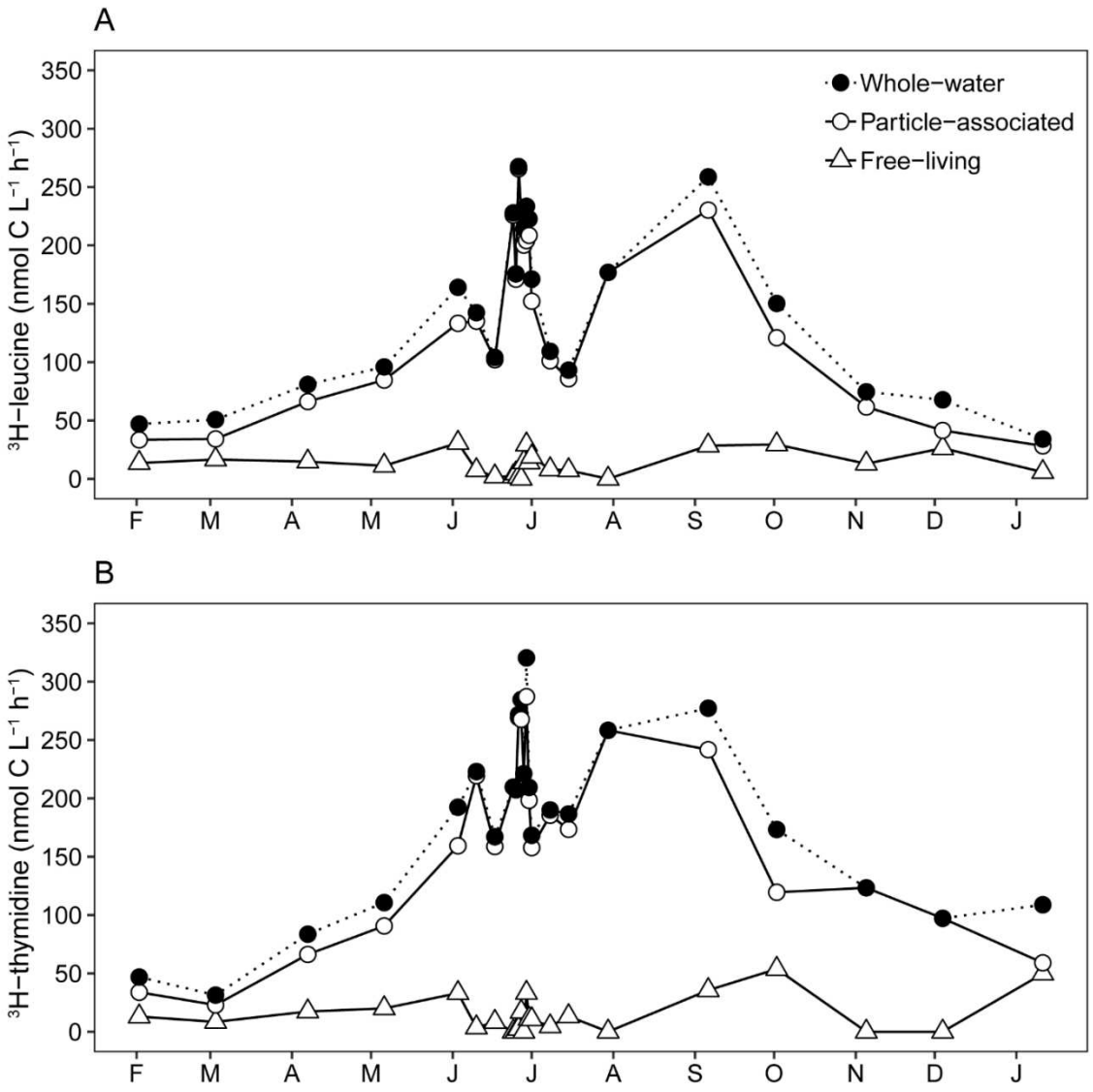

Figure 12. Rates of bacterial productivity measured from whole-water, and from particle-associated and free-living cells between February 2013 and January 2014 using (A) 3H-leucine and (B) 3H-thymidine. Particle-associated productivity was determined by subtracting free-living productivity from productivity measured in whole-water (i.e. all cells). Rates of productivity are presented as means for each date, n = 2-3.

3.4 Discussion

By evaluating the Lower Mississippi River bacterioplankton microbiome at a single river location over a range in timescales, from days up to a year, we were able to document temporal patterns of variability in its diversity and productivity. Our results allow us to assess the extent to which constant physical turnover combined with potentially fast-acting, compared to slower-acting, environmental variation drives community change.

Differences in particle-associated and free-living alpha diversity between any two days or weeks were often greater than between any two months across the sampling period. A potential explanation for this pattern may be that temporal variability among months in microbiome richness was obscured by fine-scale spatial heterogeneity (i.e. variation among replicate sub-samples). Even though the Lower Mississippi is generally well mixed there can be patchiness at a local and sub-daily scale due to its high energy and complex currents that may include gyres, eddies, and upwelling.

While differences in bacterial OTU richness were not predictable based on time interval of sampling for either component of the river microbiome, richness of OTUs was always greater on surfaces of particulate matter compared to free-living assemblages. This observation is consistent with those made previously along the length of the Mississippi in mid-summer 2013 (Payne et al. 2017), highlighting that suspended particles are important microhabitat “hotspots” for bacterial productivity (Crump et al. 1998, Ochs et al. 2010), organic matter transformations (Crump et al. 1998, Millar et al. 2015), and species richness in large river systems.

In contrast to temporal patterns in alpha diversity, we found that beta diversity of both particle-associated and free-living assemblages varied least on a daily sampling basis, more on a weekly basis, and most between samples separated by monthly intervals. At longer timescales, bacterioplankton assemblages separated by roughly six months were the most distinct from each other in composition, while those separated by more than six months up to a year became gradually more similar. This parabolic pattern aligns with temperature being an important driver of community assembly in the Lower Mississippi and other temperate aquatic environments (Crump and Hobbie 2005, Lindström, et al. 2005, Fuhrman et al. 2006, Kent et al. 2007, Gilbert et al. 2012). However, in addition to temperature, shifts in composition were related to variability in dissolved N and chlorophyll *a*, indicating that fluctuations in nutrients contribute to seasonality of the river microbiome. Thus, composition of bacterioplankton assemblages inhabiting the Lower Mississippi, and other large rivers (Crump and Hobbie 2005, Fortunato et al. 2012, 2013, Staley et al. 2015), may be predictable depending on temperature and nutrient regimes.

Patterns in composition were associated with changes in the relative abundances of bacterial taxa found to be important in other large river systems (Zwart et al. 2002, Crump and Hobbie 2005, Newton et al. 2011, Fortunato et al. 2012, 2013, Jackson et al. 2014, Read et al. 2015, Savio et al. 2015, Staley et al. 2015, Niño-García et al. 2016, Payne et al. 2017, Henson et al. 2018). The principal environmental correlate of change in proportion of most phyla in particle-associated and free-living assemblages was

temperature, to which Acidobacteria and Planctomycetes responded positively, and Bacteroidetes and Verrucomicrobia responded negatively.

Taxa identified as Actinobacteria responded positively to low river flow, and contributed to substantial differences in the free-living microbiome between mid-summer and fall. Actinobacteria were observed previously during mid-July in 2012 in major tributaries of the Mississippi (Jackson et al. 2014), and during mid-July in 2013 along a 1,300 stretch of the Mississippi itself (Payne et al. 2017), to be in much higher proportions in free-living assemblages than in the particle-associated microbiome. These studies indicate that during low flow conditions aquatic members of Actinobacteria (e.g. order Actinomycetales) are consistently prominent within free-living assemblages. These taxa may be more competitive when discharge is low due to a reduction in the immigration of allochthonous bacteria from terrestrial sources (Crump et al. 2012), and/or as a consequence of increased time in transit (Fortunato et al. 2013, Read et al. 2015, Savio et al. 2015, Niño-García et al. 2016).

The temporal patterns of variation in bacterial productivity were distinct from patterns in diversity of particle-associated and free-living assemblages. Differences in beta diversity of assemblages were maximized at around 180 days apart in sampling, regardless of the times of year being compared, while differences in bacterial alpha diversity did not vary with time interval. This is because microbiome composition varied along seasonal transitions in temperature as well as dissolved N and chlorophyll *a* concentrations, while bacterial richness oscillated unpredictably at short timescales. Bacterial productivity, in contrast, while ranging the most between cold and warm

months, was nearly identical in spring and fall, indicating the dominant influence of water temperature. However, this was the case only for particle-attached assemblages, as productivity of free-living cells did not vary with changes in the environment. Thus, our results indicate that bacterial diversity and productivity respond to different sets of drivers, resulting in different patterns of variation both within the river microbiome and across time.

The physical environment of flowing waters, and associated plankton community, are continuously in flux. Hence, at a particular riverine location, plankton assemblages could diverge rapidly in diversity and activity in response to flow-mediated immigration and emigration. Adding to the potential for rapid change in community diversity with flow rate is the reproductive potential of resident biota. Having potentially high rates of turnover, while also subject to continuous downstream flux, the bacterioplankton microbiome of a particular river location potentially could vary as much on the order of days or weeks as among months or seasons. However, unlike low-order streams and rivers, the immense volume of large rivers may buffer these systems from rapid environmental or biological variation. In that case, we would expect microbiome diversity and function to vary slowly, following seasonal or annual patterns in regional environmental drivers rather than transiently acting factors associated with random local disturbances.

In this study, we found that variation in microbiome richness was unrelated to the timescale of change in the river environment, suggesting there is a high degree of spatial variability in richness at any given moment in time. In contrast, variation in microbiome

composition, as well as particle-associated productivity, was clearly related to temporal changes in the river environment. While productivity was driven almost exclusively by water temperature, the parabolic pattern of variation in dissimilarity indicates that composition was driven by changes in temperature interacting with temporal variation in other environmental factors having a strong seasonal pattern such as dissolved N and chlorophyll *a* concentrations. Our results indicate that temporal variability in composition of the Lower Mississippi River microbiome is not random; rather, there is successional change over monthly to seasonal timescales, with gradual divergence up to 180 days, followed by gradual reassembly thereafter up to at least 360 days distance in time.

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CHAPTER IV:

TEMPORAL AND SPATIAL VARIATION IN BACTERIOPLANKTON DIVERSITY ALONG A CROSS-SECTIONAL TRANSECT OF THE LOWER MISSISSIPPI RIVER FLOODPLAIN: THE ROLE OF HYDROLOGIC CONNECTIVITY

Abstract

Large river floodplain systems are comprised of diverse aquatic habitats that vary in their flow regime. Hydrologic connection of these habitats across space and time results in exchanges of materials and organisms that is critical for maintenance of biodiversity within the river floodplain. Bacterioplankton are fundamental components of the biological structure and function of river systems. However, little is known about the distribution of these organisms among floodplain habitats in relation to environmental heterogeneity generated by hydrologic connectivity.

We documented particle-associated and free-living bacterioplankton assemblage taxonomic diversity in the Lower Mississippi River (LMR) across a transect spanning different habitats at high and low river stages. These habitats included the main channel, slack-waters in close proximity to the channel, and several large LMR-derived oxbow lakes, including one just outside the extant floodplain. We hypothesized that degree and duration of hydrologic connection to the main channel would regulate environmental conditions and formation of bacterioplankton assemblages in floodplain habitats.

Development of assemblages within the various aquatic environments aligned with the degree and timing of connection to the main channel. Particle-associated and free-living bacterioplankton assemblage composition corresponded most strongly with factors that co-varied with hydrologic connectivity such as turbidity, total suspended solids, chlorophyll *a*, and dissolved nitrogen (N) and phosphorus (P) concentrations.

The slack-water environments and bacterioplankton communities most closely resembled the river at most stages, and relatively high proportions of bacteria commonly associated with soils, such as Acidobacteria and Gammaproteobacteria, were observed in these habitats. While assemblage composition in the oxbow lakes was similar to the river after the peak hydrograph, thereafter succession of lake communities followed dissimilar trajectories than main channel or slack-water assemblages, and were dominated by cyanobacteria taxa (families Prochloraceae and Synechococcaceae). However, following isolation of these sites from the main channel for 135 days, corresponding to the lowest point in the hydrograph, the assemblages across all sites converged in composition.

We conclude that the strength and timing of hydrologic connectivity drives the pattern of succession of floodplain bacterioplankton assemblage composition. With strong hydrologic connectivity at high river stages there was nutrient loading of floodplain sites, and similarity of environmental conditions and bacterioplankton assemblage composition across the floodplain. With gradual disconnection of floodplain sites from the main channel as the river elevation dropped, there was high autochthonous productivity leading to development of bacterioplankton assemblages dominated by blooms of diverse cyanobacterial taxa, most importantly of the families Prochloraceae

and Synechococcaceae. This pattern of succession was similar in both transiently disconnected slack-water sites that separated from the main channel later in the year and oxbow lakes that separated sooner. With prolonged isolation, nutrient resources were depleted, cyanobacteria, but not necessarily algal biomass, collapsed, and the bacterioplankton assemblages converged towards a more river-like composition of principally heterotrophic taxa. In the oxbow lake outside the levees, a dissimilar successional pattern was observed, especially with respect to the timing and composition of the cyanobacterial bloom. Results indicate the important role that connection with the LMR main channel at high water plays in initiating the pattern of succession of the bacterioplankton assemblage that follows with prolonged disconnection.

4.1 Introduction

Large river alluvial floodplain systems may be comprised of diverse aquatic habitats in addition to the main channel. These may include flooded riparian forest, backwater lakes, side channels, and slack-water areas adjacent to the main channel (Junk et al. 1989, Baker et al. 1991, Tockner et al. 2000). These habitats often differ from the main channel in physical, chemical, and biological characteristics (Fremling 2005, Schramm et al. 2009), especially during low water periods when there is hydrological disconnection with the main channel (Amoros and Bornette 2002, Pongruktham and Ochs 2015). In contrast, when hydrological connection is high, habitat heterogeneity is potentially reduced with exchange of water, nutrients, other materials, and biota across the floodplain.

Synchronous development of bacterioplankton assemblages have been documented in hydrologically unconnected lakes (Kent et al. 2007) and rivers (Crump and Hobbie 2005, Crump et al. 2009) due to similar environmental characteristics of the isolated habitats, highlighting the importance of local factors in shaping community structure. From a study of the Danube river-floodplain system, Besemer et al. (2005) concluded that hydrological connectivity to the river structured environmental conditions within floodplain pools, which, in turn, structured bacterial assemblages of these habitats. In pools more frequently connected to the river, there was strong mixing with allochthonous nutrients and bacteria, which influenced patterns of succession in assemblage composition. In contrast, succession of assemblages occupying isolated pools corresponded to seasonal dynamics in temperature and autochthonous organic nutrient concentrations. Furthermore, Besemer et al. (2005) found that particle-associated and free-living assemblages in the river-floodplain were also distinct in composition and temporal dynamics. Particle-associated communities were found to be more heterogeneous compared to the free-living component, and more sensitive to fluctuations in terrestrial organic matter and algal biomass.

The Lower Mississippi River (LMR) and its floodplain consist of diverse aquatic habitats that vary in size, shape, nutrient composition, and ecological function according to the annual flow regime of the river main channel (Baker et al. 1991, Schramm et al. 2009, Pongruktham and Ochs 2015). While relationships between variability in aquatic habitats of the LMR floodplain and some biotic communities therein have been well documented (Baker et al. 1991, Pongruktham and Ochs 2015, Harrison et al. 2017, Jarrod

Sackreiter unpublished), the patterns of variation in bacterioplankton diversity and the mechanisms that drive assembly of these organisms in this system are unknown.

In this study, bacterioplankton assemblages were sampled over a six-month period along a descending river stage from aquatic habitats across a transect of the current and former LMR floodplain. These habitats included the river main channel, slack-waters in close proximity to the main channel, and several large LMR-derived oxbow lakes, including one just outside the extant floodplain. We addressed the following questions: (1) How does bacterioplankton assemblage composition vary across the LMR floodplain system? (2) What environmental parameters are associated with variation in bacterioplankton assemblage composition? (3) Is there a relationship between main channel connectivity and distribution of bacterioplankton in the current LMR floodplain?

4.2 Methods

4.2.1 Sampling sites

Eight sites were sampled across a roughly east-west transect of the LMR mainstream, its floodplain oxbow lakes, and an oxbow lake that no longer connects to the river due to the levee system (Table 4.1). These sites were chosen to represent aquatic habitats that vary in proximity to (Figure 4.1) and degree of hydrologic connection with (Figure 4.2) the LMR depending on river stage and location.

Table 8. Site location in the LMR floodplain, site identification, habitat classification, coordinates, and dates sampled.

Location	Site	Habitat	Coordinates	Dates
Mainstem	River main channel (RMC)	Main channel	34°11'07.64"N 90°53'06.94"W	05/31-10/05
	Oxbow lake outflow (OUT)	Slack-water	34°09'49.62"N 90°54'05.40"W	05/31-08/08
	Wingdam pool (WDP)	Slack-water	34°08'20.01"N 90°56'43.80"W	08/08-10/05
	Sandbar pool (SBP)	Slack-water	34°08'52.41"N 90°56'56.85"W	08/08-10/05
Floodplain	Lower Mellwood Lake (LML)	Oxbow lake	34°11'22.68"N 90°54'08.16"W	05/31-08/08
	Middle Mellwood Lake (MML)	Oxbow lake	34°12'55.56"N 90°55'55.62"W	05/31-10/05
	Middle DeSoto Lake (MDL)	Oxbow lake	34°08'17.12"N 90°51'20.18"W	05/31-10/05
Outside floodplain	Moon Lake (MOL)	Oxbow lake	34°25'18.24"N 90°30'44.58"W	05/31-10/05

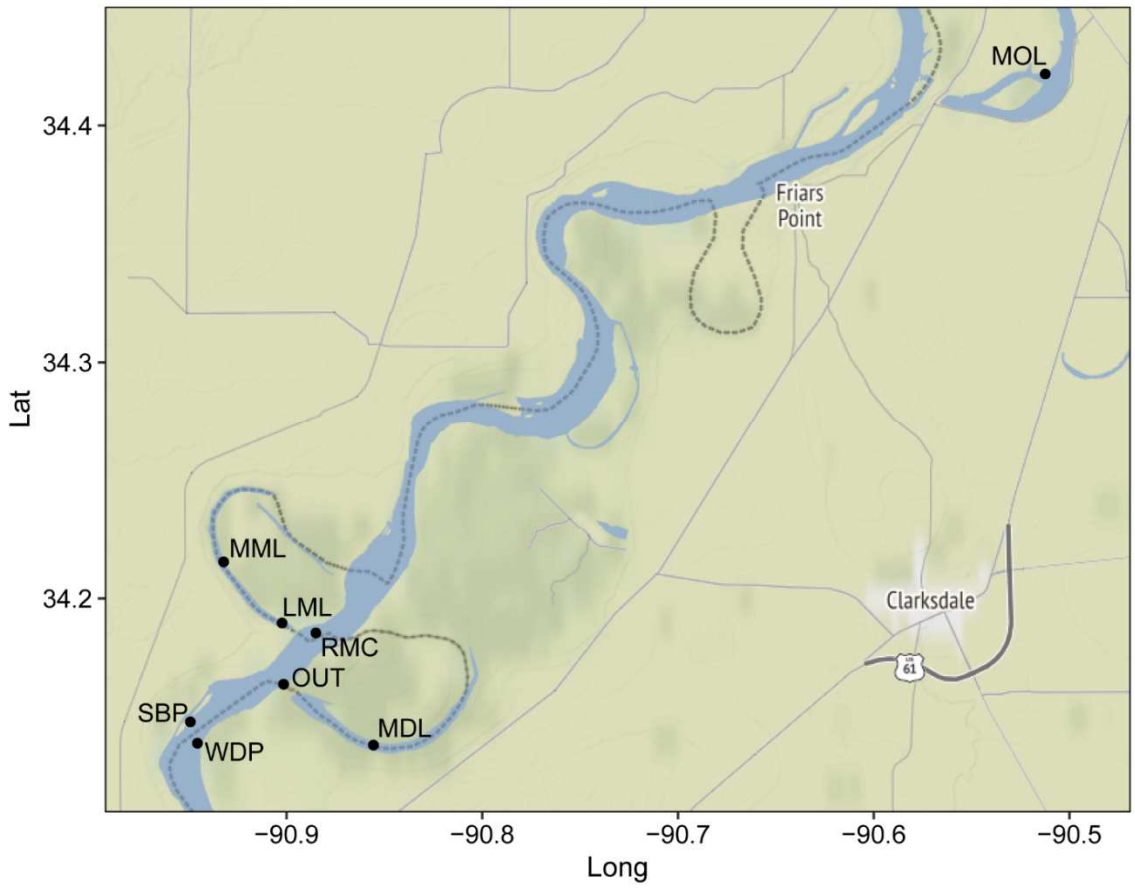

Figure 13. Map of sampling sites. Site abbreviations, location, habitat description, dates sampled are shown in Table 4.1.

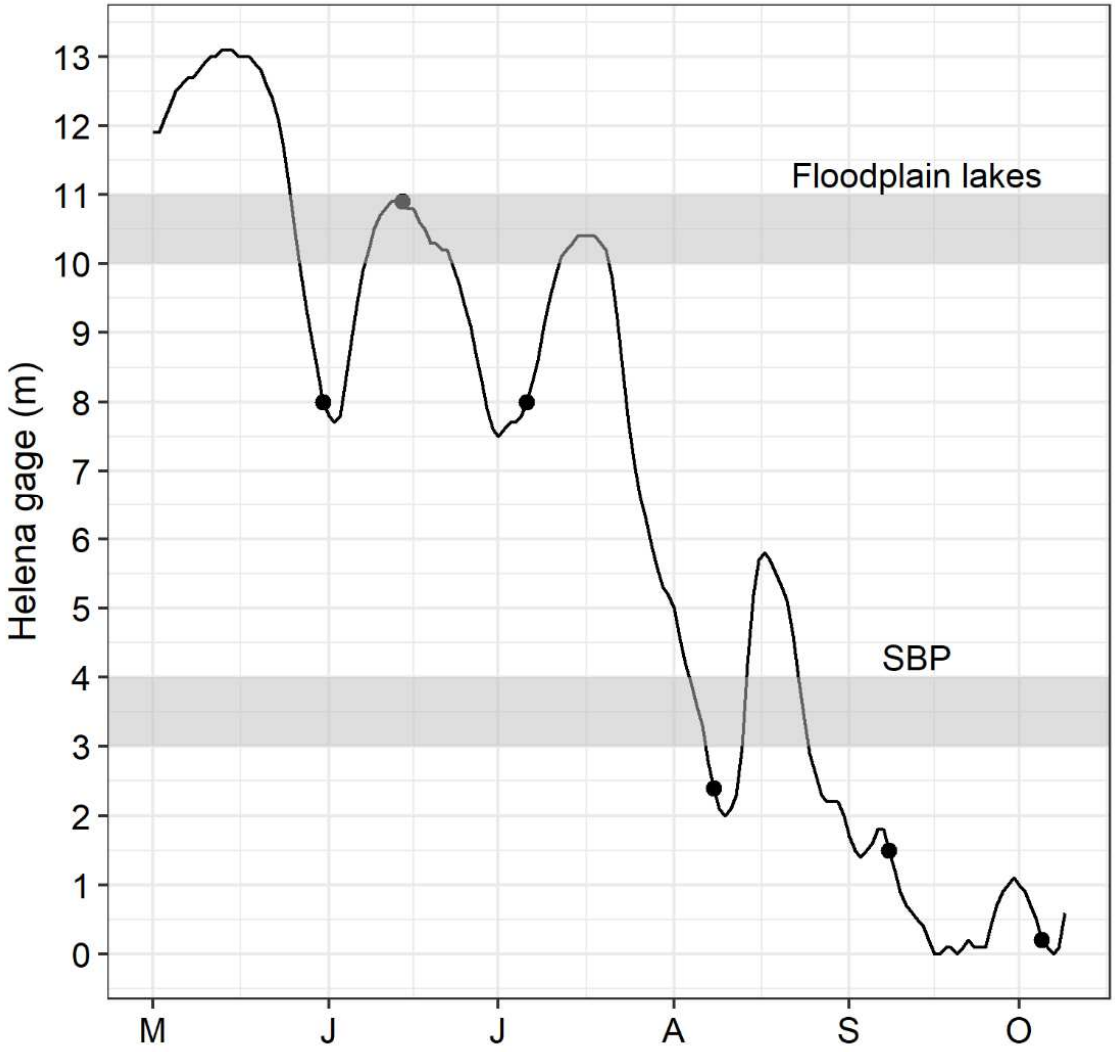

Figure 14. Lower Mississippi River stages recorded daily by the U.S. Army Corps of Engineers at Helena, Arkansas between May and October 2013. Points represent sample dates (presented in Table 4.1). Shaded areas indicate the range of critical depths of disconnection for the floodplain lakes and sandbar pool (SBP).

LMR-floodplain habitats were classified according to Baker et al. (1991). These habitats fall into two broad categories that include those that occur within or along the margin of the LMR mainstem, and those that occur outside the mainstem in the river floodplain. Mainstem habitats may have swift and deep currents such as the river main

channel, or relatively slower current “slack-waters”, such as lentic sandbar pools, which develop along the river margin as river stage drops (Baker et al. 1991). In the extant floodplain, the region between the levees, i.e. the batture, there are a variety of aquatic habitats including many oxbow lakes. Oxbow lakes are former river meanders that, depending on location, may connect strongly or weakly with the main channel according to river stage. In addition, we included in this study as an approximate control system, Moon Lake, an unconnected LMR-derived oxbow lake just outside the extant floodplain.

Sampling began on 31-May 2013 shortly after the peak hydrograph in mid-May, and lasted through periods of low river flow in October (Table 4.1, Figure 4.2). Samples were collected on an approximately monthly basis on six dates from most sites; however, sites that were occasionally inaccessible by boat, or slack-water sites that could not be differentiated from the river, were not sampled as frequently (discussed below).

Four sites were sampled in the LMR mainstem. A site near the center of the well-mixed river main channel (RMC) was chosen to characterize full river connection. Three sites that flank the main channel were chosen as representatives of slower-flowing slack-water habitats. One of these three sites was located just below the downstream connecting channel of Desoto Lake (OUT). This site was chosen to characterize a zone of frequent mixing between main channel and floodplain oxbow lake discharge. This site was inaccessible by boat at low water, and not sampled, in September and October. The other two slack-waters, a pool immediately downstream from a wingdam (WDP), and a sandbar pool (SBP), were sampled only after becoming distinguishable from the main channel in August. Although both these habitats were distinct from the main channel,

visual observations indicated currents in the wingdam pool were swifter than the sandbar pool.

We sampled two large oxbow lakes in the batture, Mellwood Lake and DeSoto Lake. These lakes are on opposite sides of the river and nearly directly across from each other. Both lakes are able to connect to the river via an upstream connecting channel through which river water entering the lake passes through the entire lake before returning to the river, and a downstream connecting channel through which river water may flow in and out with changes in stage height. Mellwood Lake was sampled at two sites, near the middle (MML) and close to the downstream connecting channel (LML). LML was accessible by boat only through August, while MML was sampled on all dates. Desoto Lake was sampled at a middle location (MDL) on all six sample dates.

Lastly, Moon Lake (MOL), a LMR-derived oxbow lake currently outside of the batture, was chosen to represent an aquatic environment that is relatively close in proximity to the LMR, but exchanges little surface water with the river main channel. Moon Lake was sampled on all study dates.

Connectivity was evaluated based on the river stage recorded at the Helena Gage (HG) near Helena, Arkansas (Figure 4.2). For sites within the batture, maximum mixing with inflowing river water was considered to have occurred on 15 May 2013 (HG = 13 m), approximately two weeks before our first samples were taken. We also referred to the HG to determine the range of critical depths at which sites lost surface water connection with the river (Figure 4.2). For example, when the river stage at Helena is 10 m or greater (HG $\geq$ 10 m), the upper connecting channels of Mellwood and DeSoto Lake receive free-

flowing water from the main channel; at HG < 10 m, there is disconnection at the upper connecting channel (Pongruktham and Ochs 2015). However, water may still enter the lower connecting channels of these lakes when the river is rising at HG > 1.6 m. There was always a surface water connection between the river and the downstream connecting channel of Desoto Lake, and the wingdam pool. The remaining slack-water site, the sandbar pool, was estimated to have lost connection with the river when the HG read between 3 and 4 m.

4.2.2 Sample collection and environmental measurements

Water was collected from all sample sites at a depth of 0.5 m using sterilized 1-L Nalgene and 500-mL Nalgene sample bottles (n = 3) for physicochemical and bacterioplankton analyses, respectively. Sampling occurred between 1100 and 1500 h on each date, and sample bottles were stored in coolers containing river water to maintain ambient temperature during transportation (1.5-3.0 h) to the laboratory for additional measurements, sample fractionation, and preservation.

Water temperature (Temp), dissolved oxygen (DO), and pH were measured on site using a YSI multiprobe instrument. In the laboratory, sub-samples (100-200 mL) were filtered through ashed 47-mm diameter Whatman GF/F filters. Filters and filtrates were preserved at -60 °C and -20 °C, respectively. Chlorophyll *a* (Chla) was extracted from filters by 90% ammonium hydroxide-buffered acetone for 24 h at 5° C on a shaking table and assayed by spectrophotometry (Wetzel and Likens 2000). Filters were also dried at 60 °C for gravimetric analysis of total suspended sediment (TSS) concentrations. Total dissolved organic C (TDOC) and total dissolved N (TDN) concentrations were

measured in filtrates using a Shimadzu Total Organic Carbon Autoanalyzer, and total dissolved P (TDP) concentrations were measured using standard spectrophotometric methods (Wetzel and Likens 2000). Turbidity (Turb) was measured using a turbidimeter (Hach model 2100A).

4.2.3 Bacterioplankton collection and DNA extraction

Bacterioplankton were collected using a serial filtration procedure outlined in Jackson et al. (2014) and Payne et al. (2017). Subsamples (100-mL) were taken from 500-mL sample bottles and passed through (<5 mm Hg vacuum) sterile Millipore 3-µm pore-size polycarbonate filters. Particles collected by this filtration are considered to represent particle-associated cells or cells >3 µm in size. After the initial filtration, filtrates were passed through sterile Millipore 0.22-µm pore-size polyethersulfone filters. Cells collected by this filtration step are assumed to be free-living and smaller (0.22-3-µm). Filters were preserved at -20 °C immediately following filtration.

Particle-associated and free-living bacterial DNA was extracted from duplicate 3-µm filters and 0.22-µm filters, respectively. Extraction was performed using PowerWater DNA Isolation Kits (MoBio, Carlsbad, CA). The V4 region of the bacterial 16S rRNA gene was amplified and sequenced using methods described previously (Stone and Jackson 2016, Payne et al. 2017), that were modified from Kozich et al. (2013). The target DNA region was amplified using PCR and the primer set Bac8f (5'-AGAGTTTGATCCTGGCTCAG-3') and Univ1492r (5'-TTCCGGTTGACCYGCCGA-3') adapted with dual-index barcodes for Illumina MiSeq next generation sequencing (Kozich et al. 2013). PCR included 30 cycles of denaturation (95 °C) for 20 s, annealing

(55 °C) for 15 s, and elongation (72 °C) for 2 min, and a final elongation (72 °C) for 10 min. Amplified DNA was normalized using SequalPrep Normalization Plates (Life Technologies, Grand Island, New York), pooled, and sequenced using an Illumina MiSeq platform located at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center.

4.2.4 Sequence processing and analysis

Sequences were denoised, aligned, and classified as operational taxonomic units (OTUs) using *mothur* software (Schloss et al. 2009), by a procedure described by Payne et al. (2017). Reads were aligned using reference V4 sequences in the SILVA rRNA database (release 119) (Pruesse et al. 2007). All unaligned sequences, and homopolymers >8 bp were discarded. Reads that were different by <2 bp were merged, and potential chimeras identified by UCHIME (Edgar et al. 2011) were removed prior to classification. Sequences were classified using the RDP database (Release 11, September 2016) (Cole et al. 2014), and non-bacterial lineages (e.g. Archaea, Eukarya, and mitochondria) were removed. Chloroplast lineages were removed in a subsequent step (see below) because the RDP database does not distinguish between cyanobacteria and chloroplast lineages at the phylum-level. All reads were then clustered into OTUs based on ≥97% similarity.

The OTU and taxonomy tables created in mothur were imported into R version 3.5.1 (R Core Team 2013), and processed further using the microbiome analysis software *phyloseq* version 1.14.0 (McMurdie and Holmes 2013). Environmental metadata was merged with OTU and taxonomy tables. OTUs classified as chloroplast lineages, and those with fewer than one read in 10% of the samples (i.e. potentially erroneous and rare

OTUs) were removed from the dataset. OTU counts were then normalized using edgeR (Robinson et al. 2010). Visual and statistical analyses were performed using the R packages *phyloseq*, *vegan* version 2.5-3 (Oksanen et al. 2018), *microbiome* version 1.9.1 (Lahit et al. 2017), and *ggplot2* version 2.1.0 (Wickham 2016).

Environmental characteristics of the river-floodplain sample sites were analyzed using principal component analysis (PCA). The PCA included all measured environmental variables (i.e. TSS, Turb, TDOC, TDN, TDP, Chla, DO, pH, and Temp). All variables were standardized to zero mean and unit variance prior to PCA analysis.

Patterns in assemblage composition among filter fractions and habitats were examined using relative abundances of bacterial taxa at broad taxonomic levels (phylum, class, family), and at a fine taxonomic level (variable OTUs recovered from between 80 and 20% of samples). Relative abundances were calculated with respect to the total number of reads obtained from samples collected on each date. Relative abundances were then averaged from replicate samples (n = 2) collected at each site.

Relationships between particle-associated and free-living beta diversity (i.e. differences in assemblage composition among sites) were visualized using distance-based redundancy analysis (dbRDA) based on Bray-Curtis dissimilarities (function "capscale" in the package *vegan*). Prior to calculating dissimilarities, OTU-count data was Hellinger transformed to give low weights to OTUs that had low and/or zero counts (Legendre and Gallagher 2001, Buttigieg and Ramette 2014). Forward selection modeling (function "ordiR2step" in the package *vegan*) was run prior to dbRDA to find the combination of variables that best explained variation in beta diversity. Initial models were run using all

parameters (i.e. TSS, Turb, TDOC, TDN, TDP, Chla, DO, pH, and Temp) that were standardized to zero mean and unit variance. A variance inflation test (function "vif.cca" in package *vegan*) identified redundant constraints, and factors with a variance inflation factor greater than 10 were removed. Marginal significance of factors was tested by analysis of variance (ANOVA). Forward selection was rerun if any variables were removed due to being redundant, or not significant. If Temp remained in a final model, this factor was used as a conditioning variable (i.e. partial dbRDA analysis).

4.3 Results

4.3.1 River hydrology and patterns in habitat environmental conditions

River stage at Helena varied between 8 and 11 meters during the initial sample dates and declined to < 1 meter in fall (Figure 4.2). Along this temporal gradient, there was substantial variation among sites in terms of their environmental characteristics (Figure 4.3). In the main channel and slack-waters, TSS concentrations and turbidity aligned with the hydrograph, with maximum values occurring in either June or July and lower values during low river flow. TSS and turbidity followed dissimilar trends among the oxbow lake habitats, declining with time at Moon Lake, but steadily increased during the study period in the floodplain lakes. DO concentrations were higher at lake sites compared to the river on most dates. DO was especially high (13 mg L^{-1}) in the floodplain lakes on 14-June, which coincided with high abundances of a cyanobacterial taxon in these habitats (*see below*). TDOC concentrations within habitats did not vary directionally with time, ranging between 5 and 12.5 mg L^{-1}. TDN and TDP concentrations in the main channel, slack-waters, and floodplain lakes trended with the

hydrograph, declining gradually over time. At Moon Lake, TDN and TDP were in the highest concentrations from May to June, and both were in much lower concentrations by August. Chla concentrations were almost always higher in the oxbow lakes compared to the river and slack-water sites. The exception to this trend occurred in August at the sandbar pool, when Chla concentrations reached 60 $\mu g\ L^{-1}$ (data not shown). In the floodplain lakes, Chla gradually increased from 20 $\mu g\ L^{-1}$ in May to 50 $\mu g\ L^{-1}$ in October. Chla concentrations at Moon Lake paralleled the pattern observed in the floodplain lakes, ranging from 10 $\mu g\ L^{-1}$ in May to 40 $\mu g\ L^{-1}$ in October. Water temperature was nearly identical across sites, ranging from 24°C in May to 32°C in September, and declined to 24°C in October. While pH was similar between the main channel and slack-waters (ranging from a pH just under 7 in May to 8 in October), this factor was often higher in lake environments, likely due to higher algal production.

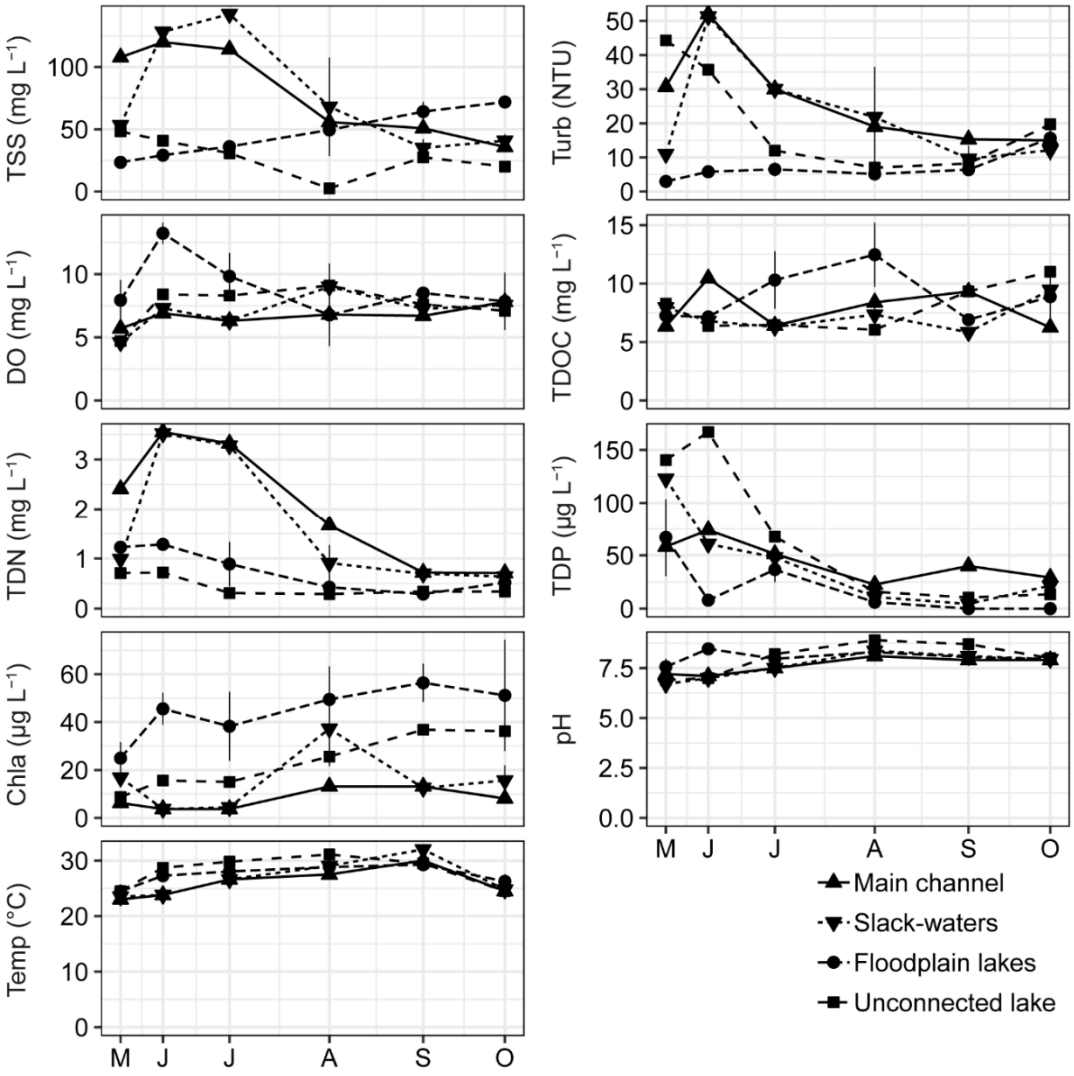

Figure 15. Environmental measurements made from Lower Mississippi river-floodplain habitats between 31-May and 5-October 2013. Measurements are presented as means for replicate samples (n = 3), except for water temperature and pH. For slack-waters and floodplain lakes, variables are presented as means and standard errors of site means per date. For the main channel and unconnected lake, variables are presented as means of replicates per date. Variable abbreviations are same as in text.

Environmental variables that best described differences between sites were examined using PCA (Figure 4.4). Results of this analysis showed strong separation of habitats along the first axis of the PCA. Turbidity, TSS, and TDN pulled in the direction of main channel samples collected between May and July and slack-water sites that were sampled in June and July. In contrast, factors related to primary productivity such as increased concentrations of Chla, DO, and higher pH levels were indicative of lake habitats during this time. TDP pulled in the direction of May and June Moon Lake samples in addition to Middle DeSoto Lake and its outflow that were sampled in May. While there was clear separation in habitat characteristics earlier in the sampling period, sites that were sampled later in the year were clustered near the center of the ordination, indicating shared environmental conditions among all habitats.

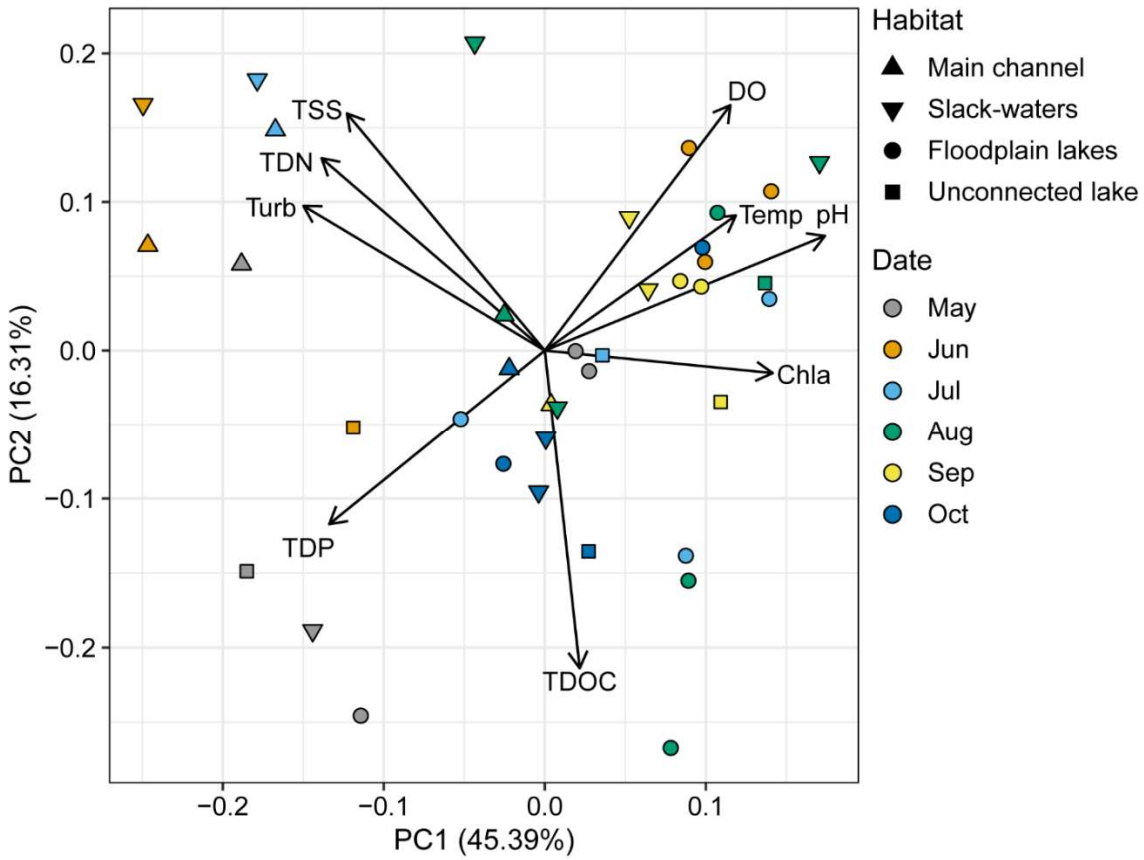

Figure 16. Principal Component Analysis (PCA) biplot showing temporal variation in habitat environmental conditions. Arrows indicate the direction and strength of the relationship of variables with samples. Variable abbreviations are same as in text.

4.3.2 Temporal and spatial patterns in bacterioplankton community composition

Relative abundances (%) of cyanobacterial taxa varied considerably among river-floodplain environments (Figure 4.5). The floodplain oxbow lakes were dominated by OTUs identified as belonging to the cyanobacteria families Prochloraceae and Synechococcaceae, whereas these taxa were nearly undetectable in the mainstem habitats for much of the period of study. While cyanobacterial taxa contributed to the most obvious differences in composition between floodplain lake and mainstem assemblages, heterotrophic taxa such as Acidobacteria and Gammaproteobacteria were in higher proportions in the river and slack-waters. At Moon Lake, Synechococcaceae was the most prominent cyanobacterial family, and Prochloraceae comprised a much lower proportion of the autotrophic assemblage at this site compared to Mellwood and DeSoto lakes.

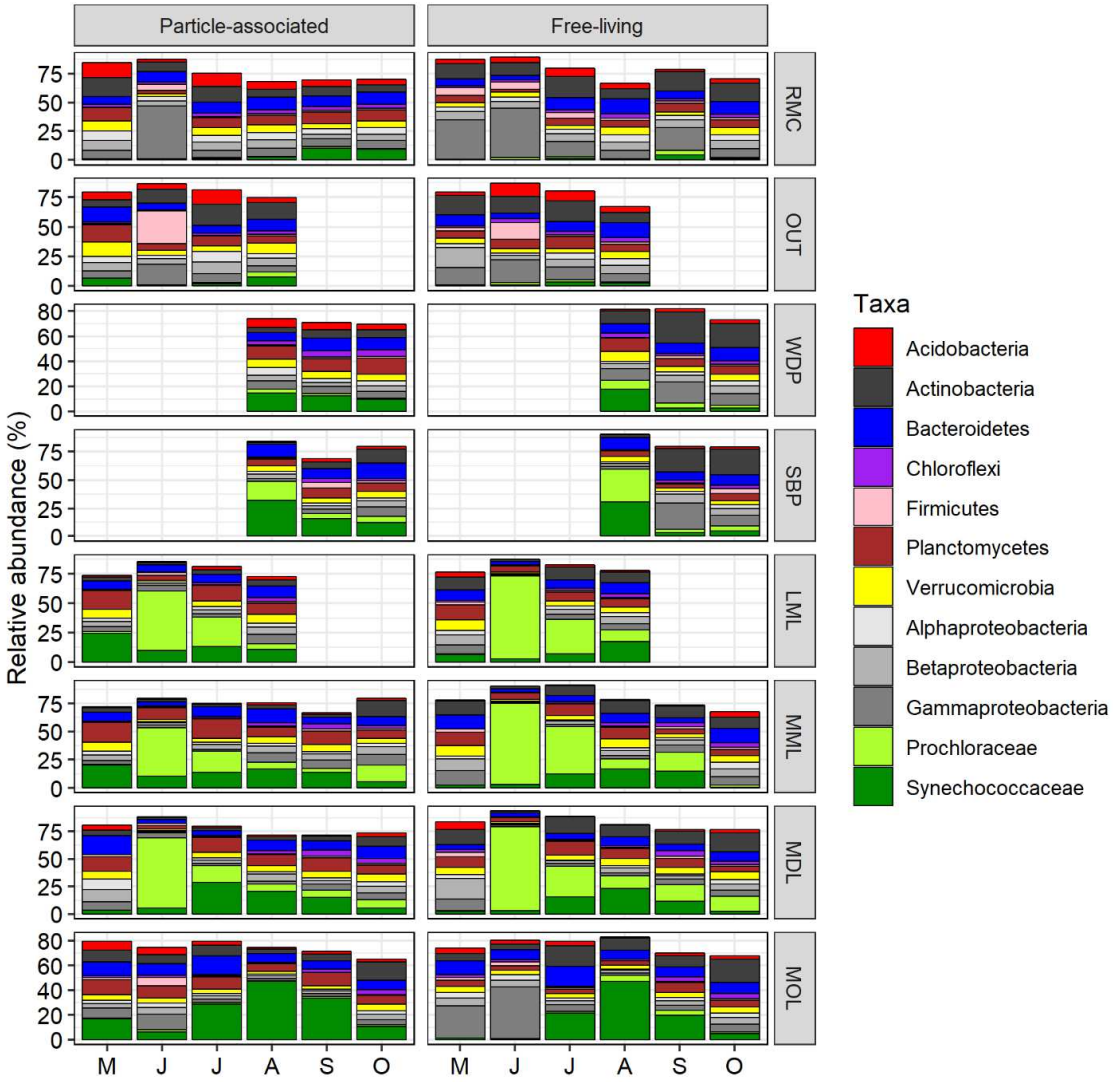

Figure 17. Spatial and temporal patterns in relative abundances (%) of bacterial taxa reads obtained from particle-associated and free-living bacterioplankton assemblages. Taxa shown are those that were ≥70% of sequences in the dataset. Proteobacteria are displayed as classes (Alpha-, Beta-, and Gamma-proteobacteria), and cyanobacteria are shown as families (Prochloraceae and Synechococcaceae). Site abbreviations are defined in Table 4.1.

Despite the major distinctions between communities described above, proportions of many heterotrophic bacterial groups were comparable across river-floodplain sites in May and again from September to October (Figure 4.5). These groups included Bacteroidetes, Planctomycetes, Verrucomicrobia, and Alpha- and Beta-proteobacteria. Furthermore, free-living assemblages across habitat types had similar proportions of Actinobacteria, but this was not the case for the particle-associated fraction.

Throughout most of the study, proportions of taxa were fairly constant within the river and slack-water habitats with a few notable exceptions (Figure 4.5). For example, on 14-June, Gammaproteobacteria dominated the river, whereas Firmicutes were in notably higher proportions at the DeSoto lake outflow site. While this event was transient for the particle-associated fraction, Gammaproteobacteria contributed to a high number of free-living reads later in the year. Interestingly, this particular group was in high abundances in free-living communities sampled from Moon Lake in May and July. Assemblages sampled from the wingdam and sandbar pool sites were mostly comprised of heterotrophic taxa in September and October. However, when the sandbar pool differentiated from the main channel in August (HG = 3 – 4 m), assemblages inhabiting this site were composed primarily of cyanobacteria related to Prochloraceae and Synechococcaceae.

In contrast to mainstem habitats, proportions of taxa varied considerably over time within lake environments (Figure 4.5). On 31-May, following the peak hydrograph in mid-May, heterotrophic taxa were the prominent members of Mellwood and DeSoto lake assemblages. However, when there was a slight upstream river connection on 14-

June (HG = 10.5 m), Mellwood and DeSoto lake communities were dominated by Prochloraceae and to a lesser extent Synechococcaceae. Proportions of these autotrophic taxa declined thereafter, with increasing proportions of heterotrophic groups. A cyanobacterial bloom occurred later in the year at Moon Lake, in contrast, with taxa identified as Synechococcaceae comprising the majority of reads obtained from August assemblages.

At a finer taxonomic resolution, the majority of Prochloraceae reads obtained from floodplain lake assemblages was attributed to a single OTU (OTU-01) (Figure 4.6). On the rising limb of the hydrograph in June, this OTU comprised between 50% and 75% of all reads, but steadily declined in proportion thereafter, with an increase in OTUs related to *Synechococcus*. While Prochloraceae dominated the floodplain oxbow lakes, an OTU (OTU-04) related to *Cyanobium* spp. was more abundant at Moon Lake. Relative abundances of specific cyanobacterial OTUs recovered from mainstem assemblages were low in comparison to the lake environments. However, at low water levels, there was a noticeable increase in relative abundances of *Cyanobium* and *Synechococcus* spp., especially in slack-waters adjacent to the river such as at the embayment site.

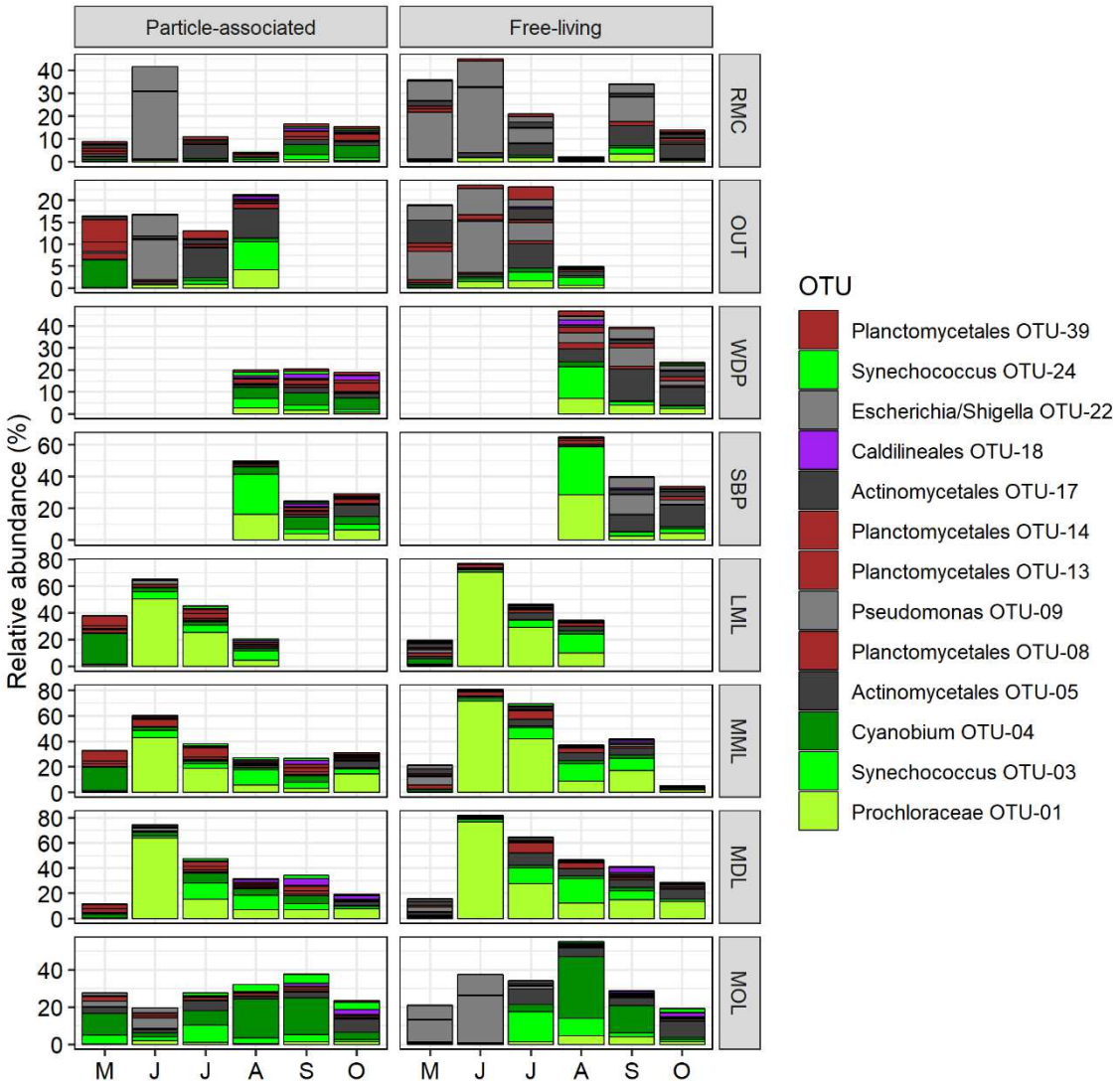

Figure 18. Spatial and temporal patterns in relative abundances (%) of variable bacterial OTUs (i.e. OTUs present in < 80% and > 20% of samples) recovered from particle-associated and free-living assemblages. Relative abundances are with respect to the total community. OTUs were identified to the lowest taxonomic resolution possible using the RDP database. Site abbreviations are presented in Table 4.1. Legend colors correspond to broader taxonomic classifications shown in Figure 4.5.

Of the non-cyanobacterial taxa, reads identified as *Pseudomonas* spp. (OTU-09) contributed to the majority of Gammaproteobacteria sequences observed at the high-water mark (14-June) during this study (Figure 4.6). As stated above, this was a transient feature of the riverine particle-associated microbiome, but repeated in the free-living fraction later in the year. This OTU was more persistent in Moon Lake, however, and abundant in this habitat from May to June. There was also a prominent OTU that co-occurred with *Pseudomonas* spp. and it was identified as *Escherichia*/*Shigella* spp. (OTU-22).

4.3.3 Temporal and spatial patterns in beta diversity and relationships to the environment

The particle-associated dbRDA ordination revealed distinct spatial and temporal patterns in community composition (Figure 4.7A). The first axis of the ordination (CAP1) explained 17% of the variation among river-floodplain assemblages. Along this axis, there was separation between mainstem and lake communities, with the greatest separation occurring between those sampled from July to September. Aligning with the major environmental and biological differences among the mainstem and floodplain habitats, TSS, turbidity, and TDN vectors were correlated with main channel and slack-water assemblages (Table 4.2). In contrast, DO, Chla, and pH were associated with floodplain lake assemblages. The second axis of the ordination (CAP2) represented temporal changes in composition, explaining 5.9% of the variation among assemblages. While TDN correlated strongly and negatively with CAP1, this factor, along with TDP, correlated positively with CAP2. As shown in the lower-left quadrant of the ordination, October samples were clustered, suggesting river-floodplain assemblages became more

similar in composition over time in response to a decline in TDN and TDP concentrations.

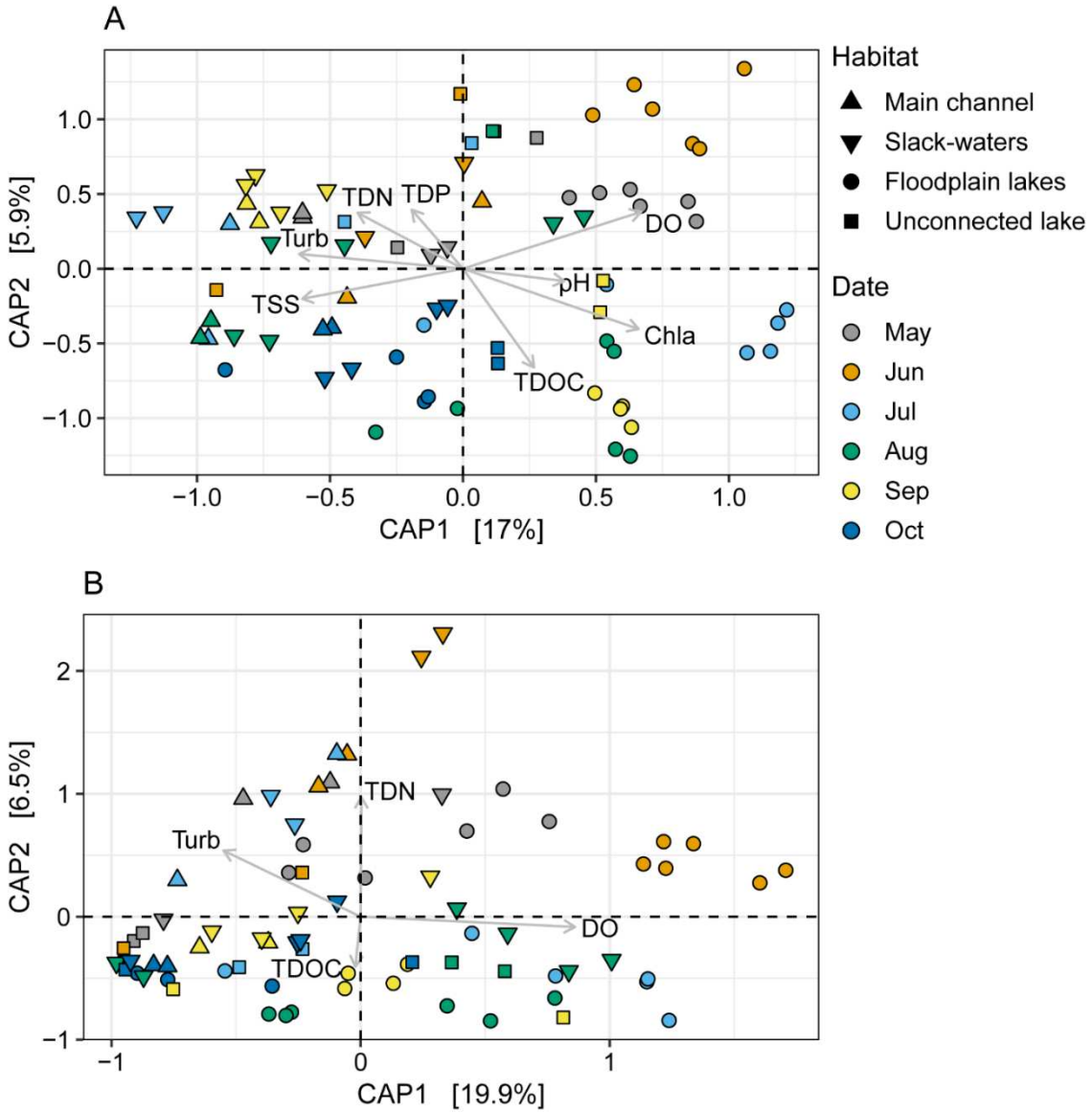

Figure 19. Distance-based redundancy analysis of Hellinger-transformed (A) particle-associated and (B) free-living OTU-count data. Habitat classifications are presented in Table 4.1. Environmental variable abbreviations are the same as in text. Statistics for dbRDAs are presented in Table 4.2.

Table 9. Correlations of environmental variables with dbRDA axes. Correlation coefficients greater than ±0.30 are shown in bold.

Fraction	Variable	CAP1	CAP2
Particle-associated	TSS	**-0.49**	-0.16
	Turb	**-0.50**	0.08
	DO	**0.54**	**0.31**
	TDOC	0.21	**-0.53**
	TDN	**-0.32**	**0.30**
	TDP	-0.15	**0.32**
	Chla	**0.53**	**-0.33**
	pH	**0.31**	-0.07
Free-living	Variable	CAP1	CAP2
	DO	**0.67**	-0.07
	TDN	0.00	**0.81**
	Turb	**-0.43**	**0.45**
	TDOC	-0.02	**-0.34**

Patterns shown in the dbRDA constructed using free-living OTUs (Figure 4.7B) resembled, but was not identical to, the trends observed for the particle-associated component. For example, there was separation between the mainstem and floodplain lake free-living assemblages along the first ordination axis (CAP1). However, this separation was more related to turbidity and DO compared to the particle-associated fraction (Table 4.2). While there also was a grouping of October free-living samples (lower-left quadrant), samples collected in September were clustered, which suggests convergence in river-floodplain free-living assemblages occurred earlier than for particle-associated assemblages. While both TDN and TDP were correlated with CAP2 of the particle-associated ordination, TDN correlated strongly and positively with CAP2 of the free-living ordination (Table 4.2). This suggests that both components of the river-floodplain

microbiome corresponded to the depletion of inorganic N, and the particle-associated fraction aligned with a decline in both TDN and TDP.

4.4 Discussion

In this study, we evaluated temporal and spatial patterns of distribution of bacterioplankton assemblages in aquatic habitats that have varying degrees of connectivity to the Lower Mississippi River. Our results allow us to assess the hydrological and environmental processes that structure the microbiome of this river-floodplain system, and potentially other large river-floodplain systems, across a gradient of high to low connectivity.

Assemblages in highly connected slack-water environments closely resembled, but were not identical to, main channel communities on most dates. Bacteria associated with soils such as Acidobacteria and Gammaproteobacteria (Crump et al. 2012) were in higher proportions in the river, the DeSoto outflow, and the wingdam site compared to the sandbar pool and lake habitats. Interestingly, OTUs closely related to *Pseudomonas* and *Escherichia*/*Shigella* spp. (Gammaproteobacteria) were in high proportions in the river and Moon Lake in May and June during relatively high river discharge. While *Pseudomonas* spp. are common in most environments, *Escherichia*/*Shigella* spp. are associated with digestive systems of warm-blooded animals. Areas that drain into Moon Lake are heavily populated with humans and cows, and *Escherichia*/*Shigella* spp. likely dispersed from these sources. The nearest source of human waste to the main channel site is approximately 100 river kilometers upstream at Memphis, TN, and non-riverine taxa such as *Escherichia*/*Shigella* are not likely to persist in the LMR over this distance

(Payne et al. 2017). However, similar to Moon Lake, the batture of the LMR is grazed frequently by local cow populations, and *Escherichia*/*Shigella* spp. may have originated from these sources.

Microbial assemblages in habitats with a lower degree of river connection were dominated by cyanobacteria, distinguishing these assemblages from those in highly connected environments. A cyanobacterial bloom occurred in Mellwood and DeSoto lakes from June to July, but this event took place later in the year in August at the Moon Lake site. Interestingly, the prominent cyanobacterial OTU found in the floodplain lakes, an OTU related to the family Prochloraceae, was nearly undetectable in Moon Lake, suggesting that environmental factors related to the LMR were drivers of growth for this taxon in oxbow lakes that are connected to the main channel. As for eukaryotic algae in floodplain oxbow lakes of the LMR (Pongruktham and Ochs 2015), members of Prochloraceae and Synechococcaceae were likely responding to higher concentrations of N due to river inputs (Reynolds et al. 2002) complemented by lower turbidity in the lentic environments. As time progressed, however, proportions of these taxa declined, which corresponded to a depletion of N (Stockner et al. 2000), higher turbidity, and increased heterotrophic bacterial abundance.

Spatial distribution of microbes can be determined by local habitat conditions (environmental selection), dispersal limitation (hydrographic barriers), or a combination of the two (Martiny et al. 2006). Bacterioplankton communities in aquatic habitats that have hydrologic connection to a river or stream undergo large shifts in composition during periods of high connectivity due to mixing with allochthonous nutrients and/or

bacteria (Besemer et al. 2005, Crump et al. 2007, 2012). Following these events, variation in community structure is associated by internal environmental selection processes, thus assemblages in isolated habitats become increasingly dissimilar from the initial mixed community.

Assemblages in the connected floodplain oxbow lakes, Mellwood and Desoto, exhibited a parallel pattern in community development, a pattern that has been noted in other aquatic systems with similar environmental characteristics (Kent et al. 2007, Crump and Hobbie 2005, Crump et al. 2009). In contrast, temporal variability at Moon Lake was not aligned with either floodplain oxbow lake, suggesting that succession of assemblages was driven by a different set of factors intrinsic to this system. A possible explanation for the asynchrony between Moon Lake and the connected oxbow lakes may be the lack of nutrient and/or bacterial inputs from the river.

Sampling began at periods of high, and oscillating, river stages from May (falling limb) to June (rising limb) to July (falling limb) and extended through low water periods from August to October 2013. Along this temporal gradient, floodplain oxbow lake communities transitioned from resembling the river microbiome in May, to being dominated by cyanobacteria in June and July, but gradually became more similar to the river later in the year. This temporal succession of LMR-connected oxbow lake assemblages may be initiated by mixing with allochthonous nutrients and/or with heterotrophic bacteria more typical of the river environment. Heterotrophic taxa are then out-competed by autotrophic prokaryotes that quickly utilize dissolved nutrients, particularly TDN. Later in the cycle, however, there is a convergence in composition as

dissolved N and P concentrations decline, and cyanobacteria abundances become similar across the river-floodplain.

In this study, we have demonstrated that hydrologic connectivity drives temporal and biogeographical patterns in bacterioplankton across aquatic habitats of the current LMR floodplain. It is clear that bacterioplankton assemblages varied with environmental distance from the river main channel, associated primarily with turbidity, chlorophyll *a*, and dissolved nutrient concentrations (e.g. N and P). Assemblages occupying LMR slack-waters mostly closely resemble the river microbiome, but new communities, comprised mostly of cyanobacteria, may bloom in these habitats with river recession. While the large floodplain lakes never fully mixed with the river, assemblages occupying these environments were relatively similar to the river at high stages and again later in the year. In a similar vein, assemblages in the unconnected oxbow lake varied independently from all other habitats for much of the year. However, even in this unconnected habitat, communities gradually became similar to the river microbiome with time. This suggests that the environmental drivers that shape bacterioplankton community composition in the LMR floodplain, in particular variation in turbidity, chlorophyll *a*, and dissolved N and P concentrations, also structure assemblages in local, but hydrologically unconnected aquatic systems.

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CHAPTER V:

CONCLUSION

A key question concerning the distribution of biodiversity is: "Why do those organisms live where they live?" Biogeography is the study of the distribution patterns of organisms over space and time (Lomolino et al. 2006). Historically, the field of biogeography has focused on describing patterns and determining processes for the distribution of macroscopic plant and animal communities. There is growing evidence that bacteria and other microorganisms also have biogeographic patterns, and similar drivers of distribution in space and over time (Martiny et al. 2006, Fierer 2008, Fierer and Lennon 2011). Many studies have discovered biogeographic patterns of bacteria inhabiting plants, humans, and other animal hosts (Turnbaugh et al. 2007, Walter and Ley 2011, Costello et al. 2012, Human Microbiome Project Consortium 2012, Christian et al. 2015), and residing in terrestrial (Kuske et al. 2002, Fierer and Jackson 2006, Lauber et al. 2009) and aquatic habitats (Fuhrman et al. 2006, Fierer et al. 2007, Lozupone and Knight 2007, Nemergut et al. 2010). However, patterns of microbial diversity in lotic aquatic ecosystems, such as stream and river networks, have received less attention (Zinger et al. 2012, Zeglin 2015). The goal of this dissertation was to describe bacterioplankton communities of the MRB and their patterns of variation in space and over time. My overarching research questions were (1) Do bacterioplankton communities

of the MRB exhibit biogeographic patterns?; (2) If so, what are the patterns of variation in space and time?; (3) What are the potential drivers of these patterns?

The main objective of the downstream study (Chapter 2) was to determine patterns of variation in bacterioplankton diversity of the Mississippi River as it flowed downriver over 1,300 river kilometers, and factors that control bacterioplankton community composition before and after confluences with large rivers of the MRB network. A corollary to this objective was to reaffirm that bacterioplankton communities in large tributaries of the MRB network (e.g. the Illinois, Missouri, Ohio, and Arkansas rivers) are distinct in composition, and are likely structured by the particular chemical and physical properties of each river (Jackson et al. 2014). In the current study, which took place a year after the study of Jackson et al. (2014), I confirmed that the large tributaries of the MRB have distinct summer bacterioplankton assemblages (Payne et al. 2017). Furthermore, I found this is true for the three major sections of the Mississippi River; the Upper, Middle, and Lower Mississippi rivers. These segments of the Mississippi had distinctive environmental profiles, and inter-river differences in bacterioplankton communities were most likely a consequence of environmental selection processes (Jackson et al. 2014, Read et al. 2015, Savio et al. 2015, Staley et al. 2013, 2014, 2015, Payne et al. 2017). Alternatively, the patterns I observed could have resulted from historical events such as geographic isolation, however, given the ease of microbial dispersal in connected aquatic systems (Finlay 2002, Martiny et al. 2006), the effect of dispersal limitation in shaping bacterioplankton communities in these rivers is probably marginal compared to environmental selection.

At a much finer spatial scale, each river of the MRB network possessed particle-associated and free-living assemblages that were dissimilar in composition (Jackson et al. 2014, Payne et al. 2017), and these discrete components most likely differed in their biogeochemical processes (Millar et al. 2015). Furthermore, richness of bacterial OTUs was consistently greater on surfaces of particulate matter compared to free-living assemblages along the length of the Mississippi River. Thus, there is spatial heterogeneity in bacterioplankton community structure, and potentially metabolic transformations, at a microhabitat level, at a regional level among large tributaries to the Mississippi River, and within the Mississippi itself (Jackson et al. 2014, Payne et al. 2017).

Proportions of dominant bacterial phyla showed little variability along the length of the Mississippi, however, there was a relatively large, but transient, shift at Memphis, Tennessee. Bacterioplankton community alpha diversity (richness of OTUs at a site) and beta diversity (variation in composition between sites), in contrast, exhibited more discernable downriver patterns. Richness of bacterial OTUs on suspended particles increased from the Upper to Lower Mississippi river, suggesting there may be broadening niche heterogeneity, and/or increased colonization of particles with downriver flow (Payne et al. 2017). Beta diversity of bacterioplankton communities traveling along the Mississippi tended to shift substantially immediately after converging with the major tributaries of the MRB network (i.e. the Illinois, Missouri, and Ohio rivers), and vary more gradually further away from these inputs, especially in the stable Lower Mississippi environment. As noted above, however, there was an abrupt change in beta diversity at Memphis. This change was associated with bacteria found in waste water (Goñi-Urriza et

al. 1999, Kulshreshtha et al. 2013), which likely originated from the city's water treatment plant. Thus, these observations indicate that along this transect of the Mississippi River bacterioplankton communities are structured by two distinct processes. At major tributary confluences or urban point sources, mass allochthonous inputs of bacterial taxa and/or their environment is the predominant driver in shaping communities, whereas at increasing distances from these confluences, local environmental selection becomes the primary influence (Payne et al. 2017).

The downstream study clearly shows that bacterioplankton communities of the MRB vary in their biogeography over a range of spatial scales, from regions (between rivers) to the Mississippi itself (within sections) to microhabitats (between assemblages). However, because bacterioplankton were collected from the MRB network over a two-week period, evaluating the timescales over which variation in communities may occur in large rivers of this network was beyond the scope of this study. Furthermore, while describing both bacterioplankton community diversity and functional attributes, such as productivity, is a desirable goal to obtain a deeper understanding of aquatic ecosystem processes, this too was outside the framework of the downstream study. Thus, the main objectives of the temporal study were to analyze bacterioplankton community composition and productivity at a single site on the LMR near Tunica, Mississippi at daily, weekly, and monthly timescales.

I found in the temporal study (Chapter 3) that consistent with observations made along the length of the Mississippi, alpha diversity was always greater on suspended particles. Furthermore, particle-attached productivity was always greater than the free-

living component which highlights that suspended particles are important microhabitat "hotspots" for bacterial abundance (Millar et al. 2015), productivity (Ochs et al. 2010), organic matter transformations (Millar et al. 2015), and diversity (Payne et al. 2017) within large rivers of the MRB.

While there were clear differences between particle-associated and free-living assemblages in both structure and function, these two components of the LMR microbiome exhibited similar patterns of temporal variation in diversity. Variation in bacterial OTU richness was often as great or greater between any two days or weeks than at monthly time intervals. In contrast, beta diversity gradually increased with time, and dissimilarity between communities was maximized at approximately 180 days between samples. Thereafter, assemblages gradually became more similar in composition, indicating that there may be an annual reassembly of bacterial taxa within the LMR. This pattern of change in composition aligned with seasonal transitions in temperature, dissolved N, and chlorophyll *a*. The timescales of change in bacterial productivity were distinct from each trend in diversity. While free-living productivity was constant and low over time, the temporal pattern in particle-attached production correlated strongly with temperature. Thus, bacterial diversity and productivity varies at discrete timescales in the LMR in response to particular sets of environmental drivers.

Variation in bacterioplankton communities of riverine systems is typically evaluated on a bi-weekly or monthly basis, therefore, information on short-term variation of these assemblages is lacking. This is a methodological shortcoming because there is potential for rapid change in community diversity that can be mediated by high

reproductive rates of individual taxa and/or frequent environmental disturbances. In the current study, daily and weekly sampling was limited to a single summer season, and the patterns observed during this time may be dissimilar from short-term variation in different seasons. However, a likely scenario is that short-term variability in diversity would be less relevant during cooler and less metabolically-active seasons. Nonetheless, by sampling at finer timescales, this study provides evidence that variation in bacterioplankton community composition and productivity within the LMR over short timescales is minor compared to monthly or seasonal temporal scales. Patterns of variation corresponded most strongly with seasonal environmental factors which further suggests that there is directional change in bacterioplankton composition and productivity over seasons as opposed to random short-term fluctuations. OTU richness, in contrast, displayed a high degree of variation at short timescales during the summer season. However, this variation in richness did not result in substantial differences in composition at daily or weekly time intervals. This suggests there may be short-term oscillations in low abundance or rare OTUs, but these events do not significantly alter overall bacterioplankton community structure. This study adds to the growing body of knowledge that seasonal variation in bacterioplankton assemblage composition is a common feature of temperate marine environments (Fuhrman et al. 2006, Gilbert et al. 2012), lakes (Kent et al. 2007, Lindström et al. 2005), large rivers (Crump and Hobbie 2005, Fortunato et al. 2012, 2013, Staley et al. 2015), and perhaps large rivers of the MRB network.

The temporal study gives a comprehensive view of the patterns of change in bacterioplankton communities occupying a single habitat-type within the LMR floodplain system: the river main channel. The LMR floodplain consists of diverse aquatic habitats that, along with their biotic residents, serve as a source and sink of nutrients that continually shift due to annual flow regimes (Ochs and Shields, *in press*). However, bacterioplankton diversity and the mechanisms that drive assembly of these organisms among these environments have not been investigated. Thus, the objective of the floodplain study was to determine the temporal and spatial scales over which bacterioplankton community composition varied among differing habitats of the LMR floodplain, and the variables that drive those changes based on the degree of connectivity with the river main channel.

The floodplain study (Chapter 4) revealed that bacterioplankton communities occupying the various floodplain habitats varied with the degree and timing of main channel connectivity. Variation in particle-associated and free-living assemblage composition was associated with factors indicative of strong river connection such as higher turbidity, total suspended solids and dissolved N concentrations, and prolonged disconnection such as increases in algal biomass and nutrient depletion (Pongruktham and Ochs 2015).

In the highly connected slack-water habitats, assemblages and their environment closely resembled, but were not identical to communities and environmental conditions of the main channel. Acidobacteria and Gammaproteobacteria, bacterial phyla associated with soils (Crump et al. 2012), were more prominent in the river and most slack-water

habitats compared to the lake sites. However, following the peak hydrograph, floodplain lake environments and assemblages, comprised mainly of heterotrophic taxa, were similar to the river presumably as a consequence of mixing with allochthonous nutrients and/or bacteria (Besemer et al. 2005, Crump et al. 2007, 2012). With disconnection from the river, backwater sites developed a distinct bacterioplankton assemblage identity dominated by cyanobacterial OTUs identified as Prochloraceae and Synechococcaceae. Proliferation of these taxa was likely a response to release of light-limitation by a decline in turbidity in combination with supplementation of dissolved N (Reynolds et al. 2002) from the river. Later in the summer, proportions of cyanobacterial taxa in backwaters decreased as dissolved N became depleted (Stockner et al. 2000) and as turbidity increased. This gave rise to new assemblages that were dominated by heterotrophic taxa, which, once again, resembled the main channel community. The overall pattern observed for bacterioplankton assemblage composition in the connected floodplain is that with high water these sites were most similar to the main channel, that upon disconnection from the main channel they became increasingly autotrophic and distinct from the river, but that after prolonged disconnection they converged with the river in dominance by heterotrophic taxa.

While communities in the connected floodplain oxbow lakes exhibited parallel development (Kent et al. 2007, Crump and Hobbie 2005, Crump et al. 2009), there was a dissimilar pattern of succession for assemblages in Moon Lake, the oxbow lake outside of the levee system, most notably regarding the timing and composition of the cyanobacterial bloom. These observations suggest that allochthonous materials delivered

to floodplain lakes during high river stages are critical to initiating succession of lake assemblages. In summary, results of this study clearly show that main channel connectivity plays an important role in structuring bacterioplankton assemblages of the LMR floodplain both in space and over time.

My research focused primarily on biogeographic patterns in bacterioplankton community composition. Bacteria are involved with metabolic processes important to the cycling of nutrients, mineralization of organic matter, and directing energy flow in aquatic ecosystems (Ducklow 2008). In future research, it is important to link aspects of bacterioplankton biogeography with patterns in biogeochemistry for a fuller understanding of bacterioplankton ecology in river systems. This could be done by combining analysis of composition with direct *in situ* measurement of biogeochemical processes and/or by transcriptomics analysis of functional genes at different places and times. Synthesizing aspects of bacterioplankton distribution and their functional processes would fill a fundamental gap in the knowledge of this critically important ecosystem.

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