

1 Nutrient dynamics and stream order influence microbial community patterns along a 2914 km
2 transect of the Mississippi River

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37 Running title: Microbial regime changes on the Mississippi River

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41 **Abstract**

42 Draining 31 states and roughly 3 million km², the Mississippi River (MSR) and its tributaries
43 constitute an essential resource to millions of people for clean drinking water, transportation,
44 agriculture, and industry. Since the turn of the 20th century, MSR water quality has continually
45 rated poorly due to human activity. Acting as first responders, microorganisms can mitigate,
46 exacerbate, and/or serve as predictors for water quality, yet we know little about their community
47 structure or ecology at the whole river scale for large rivers. We collected both biological (16S
48 and 18S rRNA gene amplicons) and physicochemical data from 38 MSR sites over nearly 3000
49 km from Minnesota to the Gulf of Mexico. Our results revealed a microbial community
50 composed of similar taxa to other rivers but with unique trends in the relative abundance patterns
51 among phyla, OTUs, and the core microbiome. Furthermore, we observed a separation in
52 microbial communities that mirrored the transition from an 8th to 10th Strahler order river at the
53 Missouri River confluence, marking a different start to the lower MSR than the historical
54 distinction at the Ohio River confluence in Cairo, IL. Within MSR microbial assemblages we
55 identified subgroups of OTUs from the phyla Acidobacteria, Bacteroidetes, Oomycetes, and
56 Heterokonts that were associated with, and predictive of, the important eutrophication nutrients
57 nitrate and phosphate. This study offers the most comprehensive view of MSR microbiota to
58 date, provides important groundwork for higher resolution microbial studies of river
59 perturbation, and identifies potential microbial indicators of river health related to
60 eutrophication.

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62

63 **Introduction**

64 By connecting terrestrial, lotic, and marine systems, rivers perform vital roles in both the
65 transport and processing of compounds in all major global biogeochemical cycles (Richey *et al.*
66 2002; Ensign and Doyle 2006; Withers and Jarvie 2008; Battin *et al.* 2009; Savio *et al.* 2015).
67 Within the carbon cycle alone, rivers collectively discharge organic carbon to the oceans at over
68 0.4 Pg C yr^{-1} (Cauwet *et al.* 2002). Perhaps more importantly, rivers are generally net
69 heterotrophic (Cole *et al.* 2007), indicating that they not only transport organic matter but host
70 active metabolic processing of it as well. Conservative estimates place heterotrophic output of
71 the world's fluvial networks (streams, rivers, and estuaries) at $0.32 \text{ Pg C yr}^{-1}$ (Cole and Caraco
72 2001; Battin *et al.* 2009). Although rivers contain a small minority of global fresh water at any
73 given moment, the considerable volumes that pass through these systems make them relevant to
74 models attempting to quantify global elemental transformations. However, despite the fact that
75 microbial functions likely play a vital role in ecosystem health for both rivers themselves and
76 their places of discharge, microbial functions in rivers remain understudied.

77 At 3734 km, the Mississippi River (MSR) is the fourth longest on earth, draining 31 U.S.
78 states and two Canadian provinces- a watershed consisting of 41% of the continental U.S (Turner
79 and Rabalais 2003; Dagg *et al.* 2004). The MSR is a major source of drinking water for many
80 U.S. cities; a critical thoroughfare for transportation, commerce, industry, agriculture, and
81 recreation; and conveys the vestiges of human activity to the Gulf of Mexico (GOM). In New
82 Orleans, the average flow rate is over $16,990 \text{ cubic meters s}^{-1}$ (cms) (Rabalais *et al.* 1996), but
83 can exceed 84,000 cms during flood stages (Singh 2012), and carries over $150 \times 10^9 \text{ kg}$ of
84 suspended sediment into the northern GOM annually (Dagg *et al.* 2004, 2005). The MSR also
85 transports considerable amounts of carbon, nitrogen and phosphorus, with average annual fluxes

86 of 2.1 TgC yr⁻¹, > 1.4 TgN year⁻¹, and > 0.14 TgP yr⁻¹, respectively (Goolsby and Battaglin 2001;
87 Cauwet *et al.* 2002; Aulenbach *et al.* 2007). Globally, the MSR represents 0.8% of the dissolved
88 organic carbon flux to the worlds oceans (Cauwet *et al.* 2002). When considering total nitrogen,
89 up to 62% can occur as nitrate (NO₃⁻) (Goolsby and Battaglin 2001). This massive discharge of
90 excess eutrophic nutrients, primarily from corn and soybean (nitrogen) and animal manure
91 (phosphorus) runoff (McIsaac *et al.* 2001; Turner and Rabalais 2004; Alexander *et al.* 2008;
92 Schilling *et al.* 2010; Duan *et al.* 2014; Staley *et al.* 2014a), fuels one of the largest marine zones
93 of seasonal coastal hypoxia in the world (Rabalais *et al.* 2002, 2007; Bianchi *et al.* 2010; Bristow
94 *et al.* 2015). Studying microbial relationships to river eutrophication will improve our
95 understanding of their contributions to either mitigating or exacerbating nutrient input.

96 Far from a homogenous jumble of organisms ferried downriver, microbial community
97 composition changes with distance from the river mouth and/or from the influence of tributaries
98 (Kolmakova *et al.* 2014; Read *et al.* 2015; Savio *et al.* 2015), resulting from altered nutrient
99 concentrations (Staley *et al.* 2014a; Van Rossum *et al.* 2015; Meziti *et al.* 2016), differing
100 dissolved organic matter (DOM) sources (Ruiz-González *et al.* 2013; Zeglin 2015; Blanchet *et*
101 *al.* 2016), and land use changes (Staley *et al.* 2014b; Van Rossum *et al.* 2015; Zeglin 2015). Past
102 studies of the Thames, Danube, Yenisei, and Columbia Rivers have found that planktonic river
103 microbial assemblages were dominated by the phyla Actinobacteria, Proteobacteria, and
104 Bacteriodetes; and taxa such as acI Actinobacteria, *Polynucleobacter* spp., GKS9 and LD28
105 *Betaproteobacteria*, CL500-29 Actinobacteria, LD12 SAR11 *Alphaproteobacteria*, and
106 *Novosphingobium* spp. (Crump *et al.* 1999; Kolmakova *et al.* 2014; Read *et al.* 2015; Savio *et al.*
107 2015).

108 Specific to the MSR, previous 16S rRNA gene amplicon and metagenomic studies have

109 demonstrated that microbial assemblages in the Minnesota portion of the river (Lake Itasca to La
110 Crescent) correlated with organic carbon concentration, total dissolved solids, and land use
111 changes (Staley *et al.* 2013, 2014a; b). Species richness increased near developed land with
112 greater concentrations of nitrate and nitrite, as opposed to sites near pastureland that had greater
113 organic carbon (Staley *et al.* 2014a). Similar to past studies of other lotic systems (Crump *et al.*
114 2012; Read *et al.* 2015; Savio *et al.* 2015), Actinobacteria and Proteobacteria increased in
115 relative abundance along the upper MSR, while OTUs belonging to Bacteroidetes decreased
116 (Staley *et al.* 2013). A follow up time-series study over two summers showed a distinct seasonal
117 signal: samples from late summer of both years were more similar to each other than those from
118 early summer (Staley *et al.* 2015). A more recent study of another portion of the MSR (above the
119 Missouri River confluence to Natchez, La.) found bacterial communities formed distinct
120 regimes, likely due to the strong influence of their respective tributaries (Jackson *et al.* 2014;
121 Payne *et al.* 2017).

122 Researchers have suggested that some of these patterns supported the application of the
123 River Continuum Concept (RCC) (Vannote *et al.*, 1980) to river microbiota. The RCC postulates
124 that as a river increases in size, the influences of riparian and other inputs will decrease as the
125 river establishes a dominant core community (Vannote *et al.* 1980). Richness will increase with
126 stream order complexity before decreasing in higher order rivers (Vannote *et al.*, 1980).
127 Therefore, as continuous systems with increasing volumes and residence times, river microbiota
128 should transition from experiencing strong influences of mass effects from terrestrial, riverbed,
129 and tributary sources to systems where species sorting plays a more important role (Crump *et al.*
130 2012; Besemer *et al.* 2013; Savio *et al.* 2015; Niño-García *et al.* 2016).

131 Accordingly, studies examining the linkages between RCC and microbial structuring
132 mechanisms (e.g. species sorting, mass effects) have found that some of the results indeed
133 supported the RCC. Specifically, numerous lotic studies found that upstream and headwaters
134 amassed high species richness, before richness decreased further downstream (Crump *et al.*
135 2012; Kolmakova *et al.* 2014; Savio *et al.* 2015; Niño-García *et al.* 2016). This follows the
136 expected pattern outlined by the RCC where early stream and river microbial communities are
137 heavily influenced by a large input of allochthonous organisms from sediment and groundwater
138 into the river network. As the rivers progressed, such mass effects likely heeded to species
139 sorting, a process requiring an increased water residence time to allow for the selection of
140 species based on local environmental conditions (Crump *et al.* 2012; Savio *et al.* 2015; Niño-
141 García *et al.* 2016). These types of river modulations, influenced by hydrology and
142 environmental conditions, have correlated with decreased species richness and increased core
143 community membership (the taxa found consistently throughout the entire river) in multiple
144 systems (Crump *et al.* 2012; Besemer *et al.* 2013; Savio *et al.* 2015; Niño-García *et al.* 2016). An
145 analyses of an upper portion of the MSR provided evidence for the importance of species sorting
146 (Staley *et al.* 2015). However, in a study of the Thames River, researchers found no association
147 between microbial assemblages and any measured variables other than residence time, leading
148 them to suggest that ecological succession was the primary driver of microbial community
149 composition (Read *et al.* 2015). Furthermore, a 1300 km transect of the lower MSR indicated
150 that richness actually increased instead of decreasing in particle-associated, and to a lesser
151 extent, free-living, communities, suggesting that the MSR may have fundamentally different
152 mechanisms driving microbial diversity than other rivers (Payne *et al.* 2017).

153 Complicating matters, particle-associated communities in rivers (frequently defined as
154 those found on filters of $> \sim 3 \mu\text{m}$ remain distinct from their free-living counterparts (Crump *et*
155 *al.* 1999; Riemann and Winding 2001; Jackson *et al.* 2014), potentially due to increased
156 production rates from access to readily obtainable carbon (Crump and Baross 1996; Crump *et al.*
157 1998, 1999). Typical river microorganisms associated with particles include OTUs related to the
158 Bacteroidetes clades *Flavobacteria* and *Cytophaga*, Planctomycetes, *Rhizobium* spp., and
159 *Methylophilaceae* spp. (Crump and Baross 1996; Allgaier and Grossart 2006; D'Ambrosio *et al.*
160 2014; Jackson *et al.* 2014). However, denoting consistent trends in particle community
161 composition must be tempered by recent evidence suggesting substrate availability and chemical
162 queues may trigger organisms to switch between free-living and particle-associated lifestyles
163 (Grossart 2010; D'Ambrosio *et al.* 2014). Rivers constitute complex and highly dynamic
164 ecosystems from a metacommunity perspective.

165 Although important insights have been gained from recent research on portions of the
166 MSR (Staley *et al.* 2013, 2015, 2016; Payne *et al.* 2017), microbiological transects at the whole-
167 river or catchment scale have yet to be completed. This study aimed to *i*) compare the structure
168 and abundance of size-fractionated MSR microbial community populations to those in other
169 rivers, *ii*) examine within-river heterogeneity of microbial communities, and *iii*) identify MSR
170 microorganisms most strongly associated with eutrophication- all at a near whole-river scale, for
171 both prokaryotes and microscopic eukaryotes. During the fall of 2014, we completed the most
172 extensive microbiological survey of the Mississippi River to date with a continual rowed transect
173 over 70 days. Rowers from the adventure education non-profit OAR Northwest collected
174 samples from Minneapolis, MN to the Gulf of Mexico (2918 km) (Fig. 1A). Our findings expand
175 the current information available on microbial assemblages in major lotic ecosystems; further

176 delineate the relationships between microbial structure and stream order, nutrients, and volume;
177 and identify MSR taxa predictive of the eutrophication nutrients nitrate and phosphate.

178

179 **Materials and Methods**

180

181 *Sampling and Cell Counts*

182 We used rowboats and a simple filtration protocol (Supplementary Information) to collect
183 water from 39 sites along a continual-rowed transect of the MSR, starting in Lake Itasca and
184 ending in the GOM, over 70 days from September 18th to November 26th, 2014. Sites were
185 chosen to be near major cities and above and below large tributaries. After some samples were
186 removed due to insufficient sequence data, contamination, or incomplete metadata (see below),
187 the final usable set of samples included 38 sites starting at Minneapolis (Fig. 1A, Table S1,
188 General Information). Most sampling occurred within the body of the river, although due to
189 safety issues, three samples were collected from shore (Table S1, General Information). We
190 collected duplicate samples at each site, but because separate rowboat teams frequently collected
191 these sometimes several dozen meters apart, they cannot be considered true biological replicates
192 and we have treated them as independent samples.

193 At each site, we filtered 120 mL of water sequentially through a 2.7 μm GF/D filter
194 (Whatman GE, New Jersey, USA) housed in a 25 mm polycarbonate holder (TISCH, Ohio,
195 USA) followed by a 0.2 μm Sterivex filter (EMD Millipore, Darmstadt, Germany) with a sterile
196 60 mL syringe (BD, New Jersey, USA). We refer to fractions collected on the 2.7 μm and 0.22
197 μm filters as $> 2.7 \mu\text{m}$ and 0.2-2.7 μm , respectively. Flow-through water from the first 60 mL
198 was collected in autoclaved acid-washed 60 mL polycarbonate bottles. Both filters were wrapped

199 in parafilm, and together with the filtrate, placed on ice in Yeti Roadie 20 coolers (Yeti, Austin,
200 TX) until shipment to LSU. Further, 9 mL of whole water for cell counts was added to sterile 15
201 mL Falcon tubes containing 1 mL of formaldehyde and placed into the cooler. The final cooler
202 containing samples from sites P-AI had substantial ice-melt. Though our filters were wrapped in
203 parafilm, we processed melted cooler water alongside our other samples to control for potential
204 contamination in these filters. Given that some of our samples were expected to contain low
205 biomass, we also included duplicate process controls for kit contamination (Salter *et al.* 2014;
206 Weiss *et al.* 2014) with unused sterile filters.

207 Flow-through 0.2 μm filtered water from each collection was analyzed for SiO_4 , PO_4^{3-} ,
208 NH_4^+ , NO_3^- , and NO_2^- ($\mu\text{g/L}$) at the University of Washington Marine Chemistry Laboratory
209 (<http://www.ocean.washington.edu/story/Marine+Chemistry+Laboratory>). Aboard-rowboat
210 measurements were taken for temperature and turbidity. We determined turbidity by deploying a
211 secchi disk (Wildco, Yulee, FL), while drifting with the current so the line hung vertically. It was
212 lowered until no longer visible, then raised until just visible, and measured for its distance below
213 the waterline. We then calculated secchi depth from the average of two measurements.
214 Temperature was measured with probes from US Water Systems (Indianapolis, IN), rinsed with
215 distilled water between samples. Samples for cell counts were filtered through a 2.7 μm GF/D
216 filter, stained with 1x Sybr Green (Lonza), and planktonic cells were enumerated using the
217 Guava EasyCyte (Millipore) flow cytometer as previously described (Thrash *et al.* 2015).

218 Throughout the transect, seven cooler exchanges occurred to ensure samples were not
219 exposed to extended durations at *in situ* temperatures. Cooler temperatures were monitored with
220 HOBO loggers (Onset, Bourne, MA) to ensure that samples stayed at $\leq 4^\circ\text{C}$. On average, a
221 sample spent 7.6 days (median = 6 days, range=1 day minimum, 16 days maximum) at $\leq 4^\circ\text{C}$

222 before delivery to LSU. Though samples were not frozen immediately, previous research has
223 provided evidence that soil and water samples stored at 4°C, and for different durations (≥ 14
224 days), did not show significant alterations to most community relative abundances, structure, or
225 composition (Lauber *et al.* 2010; Rubin *et al.* 2013; Tatangelo *et al.* 2014).

226

227 *DNA extraction and Sequencing*

228 DNA was extracted from both filter fractions and controls using a MoBio PowerWater DNA kit
229 (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol with one minor
230 modification: in a biosafety cabinet (The Baker Company, Stanford, ME), Sterivex filter
231 housings were cracked open using sterilized pliers and filters were then removed by cutting
232 along the edge of the plastic holder with a sterile razor blade before being placed into bead-
233 beating tubes. DNA was eluted with sterile MilliQ water, quantified using the Qubit2.0
234 Fluorometer (Life Technologies, Carlsbad, CA), and stored at -20° C. Bacterial and archaeal
235 sequences were amplified at the V4 region of the 16S rRNA gene using the 515f and 806r primer
236 set (Caporaso *et al.* 2012), and eukaryotic sequences from the V9 region of the 18S rRNA gene
237 using the 1391r and EukBR primer set (Amaral-Zettler *et al.* 2009). Amplicons were sequenced
238 on an Illumina MiSeq as paired-end 250 bp reads at Argonne National Laboratory. Sequencing
239 of the 16S and 18S rRNA gene amplicons resulted in 13,253,140 and 13,240,531 raw sequences,
240 respectively.

241

242 *Sequence Analysis*

243 We analyzed amplicon data with Mothur v.1.33.3 (Schloss *et al.* 2009) using the Silva v.119
244 database (Pruesse *et al.* 2007; Quast *et al.* 2013). Briefly, 16S and 18S rRNA gene sequences

245 were assembled into contigs and discarded if the contig had any ambiguous base pairs, possessed
246 repeats greater than 8 bp, or were greater than 253 or 184 bp in length, respectively. Contigs
247 were aligned using the Silva rRNA v.119 database, checked for chimeras using UCHIME (Edgar
248 *et al.* 2011), and classified also using the Silva rRNA v.119 database. Contigs classified as
249 chloroplast, eukaryotes, mitochondria, or “unknown;” or as chloroplast, bacteria, archaea,
250 mitochondria, or “unknown;” were removed from 16S or 18S rRNA gene data, respectively. The
251 remaining contigs were clustered using the `cluster.split()` command into operational taxonomic
252 units (OTUs) using a 0.03 dissimilarity threshold ($OTU_{0.03}$). After these steps, 146,725 and
253 131,352 OTUs remained for the 16S and 18S rRNA gene communities, respectively.

254

255 *Sample quality control*

256 To evaluate the potential for contamination from extraction kits, cooler water in the last set of
257 samples, or leaking/bursting of pre-filters, all samples were evaluated with hierarchical
258 clustering and NMDS analysis. Hierarchical clustering was performed in R using the *hclust*
259 function with methods set to “average”, from the *vegan* package (Oksanen *et al.* 2015). Samples
260 were removed from our analysis if they were observed to be outliers in both the NMDS and
261 hierarchical clustering such that they grouped with our process controls. The process and cooler
262 water controls were extreme outliers in both, as was sample L2 (Fig. S1, S2). Sterivex and
263 prefilter samples generally showed strong separation with the exception of three 16S rRNA gene
264 samples- STER X2, W2, S2 (Fig. S1, S2). The only other samples that were removed were due
265 to missing chemical data (Lake Itasca1-2, A1-2) or failed sequencing (16S STER Af1; 16S PRE
266 S2, X2; 18S PRE O1). Not including process or cooler water controls, 152 samples were
267 sequenced each for prokaryotic and eukaryotic communities. After these QC measures, 144 and

268 149 samples remained in the analyses from the 16S and 18S rRNA gene amplicons, respectively.
269 Further, to control for potential contaminants, any OTU with greater 20 reads in the process or
270 cooler controls was removed from the data set. 146,725 and 131,327 OTUs remained after these
271 steps for 16S and 18S rRNA gene communities, respectively.

272

273 *Alpha and Beta Diversity*

274 OTU_{0.03} analyses were completed with the R statistical environment v.3.2.1 (R Development
275 Core Team 2015). Using the package PhyloSeq (McMurdie and Holmes 2013), alpha-diversity
276 was first calculated on the non-normalized, unfiltered OTUs using the “estimate richness”
277 command within PhyloSeq, which calculates six alpha diversity metrics (McMurdie and Holmes
278 2013). After estimating alpha diversity, potentially erroneous rare OTUs, defined here as those
279 without at least two sequences in 20% of the sites, were discarded. After this filter, the dataset
280 contained 950 and 724 16S and 18S rRNA gene OTUs, respectively. For site-specific
281 community comparisons, OTU counts were normalized using the package DESeq2 (Love *et al.*
282 2014) with a variance stabilizing transformation (Learman *et al.* 2016). Beta-diversity between
283 samples was examined using Bray-Curtis distances via ordination with non-metric
284 multidimensional scaling (NMDS). Analysis of similarity (ANOSIM) was used to test for
285 significant differences between groups of samples (e.g lower versus upper MSR) using the
286 *anosim* function in the *vegan* package (Oksanen *et al.* 2015). The influence of Hellinger
287 transformed environmental parameters on beta-diversity was calculated in R with the *envfit*
288 function. Relative abundances plots were made using the R package *ggplot2*. Linear and non-
289 linear regressions were made using the command *stat_smooth()* with the flag “level=0.95” and
290 method = “lm” or “loess” for linear and non-linear regressions, respectively.

291
292 *Network analyses and modeling*
293 To identify specific OTUs with strong relationships to environmental parameters (e.g. turbidity,
294 NO_3^-), we employed weighted gene co-expression network analysis (WGCNA) (Langfelder and
295 Horvath 2008) as previously described for OTU relative abundances (Guidi *et al.* 2016). First, a
296 similarity matrix of nodes (OTUs) was created based on pairwise Pearson correlations across
297 samples. This was transformed into an adjacency matrix, which represents the strength at which
298 two OTUs within the matrix are connected (adjacency). To do this, the similarity matrix was
299 raised to a soft threshold power (p ; $p = 6$ for 16S and 18S $> 2.7 \mu\text{m}$, $p = 4$ for 16S 0.2-2.7 μm , p
300 $= 9$ for 18S 0.2-2.7 μm) that maximizes OTU connections with others and ensures scale-free
301 topology fit. Submodules of highly co-correlating OTUs were assigned a color (e.g. yellow,
302 green, turquoise) as defined with a topological overlap measure and hierarchical clustering,
303 which identified submodules based on an OTU's weighted correlations to other OTUs within and
304 outside the network. Each submodule, represented by an eigenvalue, was pairwise Pearson
305 correlated to individual Hellinger-transformed environmental measurements (Figs. S7-10A). To
306 explore the relationship of submodule structure to these parameters, OTUs within the submodule
307 were plotted using their individual correlation to the parameter of interest (y-axis, here nitrate or
308 phosphate) and their submodule membership (x-axis), defined as the strength of an OTU
309 (number of connections) to other OTUs within the submodule (Figs. S7-10B, D). Strong
310 correlations between submodule structure and an environmental parameter facilitate
311 identification of OTUs that are highly correlated to that parameter. To evaluate the predictive
312 relationship between a submodule and a parameter, we employed partial least square regression
313 (PLS) analysis. PLS maximizes the covariance between two parameters (e.g., OTU abundances

314 and nitrate concentration) to define the degree to which the measured value (OTU abundance)
315 can predict the response variable (nutrient concentration). The PLS model was permuted 1000
316 times and Pearson correlations were calculated between the response variable and leave-one-out
317 cross-validation (LOOCV) predicted values. Modeled values were then compared with measured
318 values to determine the explanatory power of the relationships (Figs. S7-10C, E). Relative
319 contributions of individual OTUs to the PLS regression were calculated using value of
320 importance in the projection (VIP) (Chong and Jun 2005) determination. PLS was run using the
321 R package *pls* (Mevik and Wehrens 2007), while VIP was run using additional code found here:
322 <http://mevik.net/work/software/VIP.R>. Though other environmental parameters had strong
323 Pearson correlations to submodules, only submodules with strong, positive correlations to
324 measures related to eutrophication were selected, in accordance with our study objectives, as
325 these were most likely to provide candidate taxa with remediation potential.

326

327 *Accession numbers*

328 Community 16S and 18S rRNA gene sequence fastq files are available at the NCBI Sequence
329 Read Archive under the accession numbers: SRR3485674 - SRR3485971 and SRR3488881 -
330 SRR3489315, respectively.

331

332 *Code Availability*

333 All code used for Mothur, SeqENV, PhyloSeq, WGCNA, and PLS regression analyses can be
334 found on the Thrash lab website (<http://thethrashlab.com/publications>) with the reference to this
335 manuscript linked to “Supplemental Information”.

336

337 **Results**

338 Using rowboats and a simple syringe-based filtration protocol, we measured 12 biological,
339 chemical, and physical parameters (e.g. 16S and 18S rRNA gene communities, NH_4^+ , river
340 speed, etc.) from 38 sites along a 2918 km transect of the MSR (Fig. 1A). River order increases
341 dramatically at the Missouri confluence [eighth to tenth Strahler order (Pierson *et al.* 2008)],
342 which corresponded to overall discharge (Fig. 1A) and beta diversity changes discussed below.
343 Therefore, we refer to this juncture as the separator between the upper (0 km – 1042 km, Sites A-
344 S) and lower MSR (1075-2914 km, Sites T-Al). Within the upper MSR, NO_3^- , PO_4^{3-} , and NO_2^-
345 were variable but generally increased downriver until peak concentrations near the confluences
346 of the Illinois and Missouri Rivers. This gave way to lower and more consistent concentrations
347 along the lower MSR (Fig. 1B). Turbidity (inversely related to secchi disk visibility) increased
348 steadily downriver to a maximum near the Illinois and Missouri River confluences (1042 km,
349 Site S) (Fig. 1B, Secchi Disk), then trended downwards for the rest of the transect. Planktonic (<
350 $2.7 \mu\text{m}$) cell counts varied between 1 and $3 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ in the upper MSR, and decreased to
351 high $10^5 \text{ cells} \cdot \text{mL}^{-1}$ in the lower MSR (Fig. 1B, Planktonic Cell Counts). Water temperature
352 ranged from 19°C (133km, Site E) to 11.7°C (2552 km, Site Ag), and river speed, excluding
353 three sites sampled from shore, was between 5.5 mph at Site Y and 0.4 mph (597 km, Site L)
354 (Table S1, General Information). Spearman rank correlations of the measured environmental
355 parameters showed strong positive correlations between nitrate, phosphate, distance, and
356 increased turbidity; while nitrate and phosphate both strongly correlated negatively to water
357 temperature and river speed (Table S1, Spearman Rank).

358

359 *Bacterial and archaeal communities*

360 We observed a clear distinction between the 0.2-2.7 μm and $> 2.7 \mu\text{m}$ 16S rRNA gene
361 communities (ANOSIM $R = 0.65$, $P = 0.001$) (Fig. S1A). Both size fractions also showed
362 significant community separation between sites above and below the Missouri River confluence
363 (ANOSIM, $> 2.7 \mu\text{m}$: $R = 0.44$, $P = 0.001$; 0.2-2.7 μm : $R = 0.48$, $P = 0.001$) (Fig. 2A, B), that the
364 Eukaryotic fractions mirrored (below)(Fig. 2C, D). Overall richness was significantly higher in
365 the lower vs. upper rivers (Table S1, Richness comparisons). Taken separately, most
366 measurements indicated that richness increased with river distance in the upper and lower rivers,
367 and this trend was common for both the $> 2.7 \mu\text{m}$ and 0.2-2.7 μm fractions (Fig. S3A, B).
368 Evenness measurements had less consistent trends.

369 Phosphate and turbidity had the highest correlation with the separation between the upper
370 and lower $> 2.7 \mu\text{m}$ communities ($R^2 = 0.58$, $R^2 = 0.54$, respectively), with water temperature (R^2
371 $= 0.52$) also contributing (Table 1). At an OTU level, taxa related to the acI clade
372 (Actinobacteria) and unclassified *Bacillaceae* ($R^2 > 0.77$, $P = 0.001$) contributed most to the
373 separation between the upper and lower > 2.7 communities, with OTUs related to the *Bacillales*,
374 *Gemmatimonadaceae*, *Peptococcaceae*, and *Micromonosporaceae* clades also a factor ($R^2 >$
375 0.70 , $P = 0.001$) (Table 2). For the 0.2-2.7 μm fraction, nitrate were the strongest correlating
376 environmental factors with the distinction between upper and lower communities ($R^2 = 0.443$),
377 although phosphate, turbidity, and water temperature ($R^2 > 0.385$ for each) also contributed
378 (Table 1). OTUs related to *Flavobacterium* and an unclassified Bacterium (closest NCBI BLAST
379 hit *Acidovorax* sp., KM047473), most strongly contributed to the separation between the 0.2-2.7
380 μm communities ($R^2 > 0.503$, $P = 0.0001$) (Table 2). Other important OTUs driving the
381 separation between upper and lower communities belonged to the clades Bacteroidetes,
382 *Microbacteriaceae*, *Clostridiales*, and *Holophagaceae* ($R^2 > 0.49$, $P = 0.0001$) (Table 2).

383 At the phylum level, Proteobacteria, Actinobacteria, and Bacteroidetes dominated
384 bacterial communities in both fractions (Figs. 3A and B) (Table S1, 16S OTU table norm.).
385 Proteobacteria abundance in the > 2.7 μm fraction fluctuated over the course of the transect (Fig.
386 3A), whereas their 0.2-2.7 μm counterparts generally increased in relative abundance downriver
387 (Fig. 3B). 0.2-2.7 μm Bacteroidetes and Actinobacteria generally decreased in the upper river
388 and stabilized in the lower river. These phyla showed considerable abundance variation in the >
389 2.7 μm fraction. Cyanobacteria in the > 2.7 μm fraction negatively correlated with increased
390 turbidity (Spearman rank = 0.67), consistent with lower irradiance (Fig. 3A). Both > 2.7 μm and
391 0.2-2.7 μm Acidobacteria increased in abundance downriver and positively correlated with river
392 distance (Wilcoxon single ranked test, $P = < 0.01$) (Fig. S4A, B). Within the 0.2-2.7 μm fraction,
393 the five most abundant OTUs were classified as a LD12 (OTU11; Proteobacteria), two acI clade
394 OTUs (OTU4, OTU7; Actinobacteria), a *Limnohabitans* sp. (OTU2; Proteobacteria), and a LD28
395 (OTU8; Proteobacteria) (Table S1, 16S OTU table norm.). Comparatively, an unclassified
396 *Methylophilaceae* (OTU1; Proteobacteria), a *Planktothrix* sp. (OTU12; Cyanobacteria), a NS11-
397 12 marine group (OTU21; Bacteroidetes), an *Candidatus Aquirestis* sp. (OTU17; Bacteroidetes),
398 and an unclassified *Sphingobacteriales* (OTU25; Bacteroidetes) were the most abundant OTUs
399 in the > 2.7 μm fraction (Table S1, 16S OTU table norm.). Archaea occurred at much lower
400 relative abundances: we found only eight OTUs belonging to the phyla Euryarchaeota and
401 Thaumarchaeota, collectively. These OTUs were classified as *Methanobacterium* sp. (OTU714,
402 1968) and soil Crenarchaeotic Group (OTU370, 389, 983, and 1253), *Candidatus*
403 Nitrosoarchaeum sp. (OTU1093) from the phylum Euryarchaeota, and an unclassified
404 Thaumarchaeota (OTU2951) from the phylum Thaumarchaeota (Table S1, 16S OTU table
405 norm.). Overall, Thaumarchaeota increased in abundance along the transect more in the > 2.7 μm

406 fraction compared to the 0.2-2.7 μm fraction (Fig. S4A, B). In both fractions, we only detected
407 Euryarcheota at very low abundances. Importantly, the primers used in this study may miss some
408 archaeal taxa (Parada *et al.* 2015).

409

410 *Microbial eukaryotic communities*

411 Eukaryotic communities, observed via the 18S rRNA gene, also showed a significant separation
412 between $> 2.7 \mu\text{m}$ and 0.2-2.7 μm fractions (ANOSIM $R = 0.689$, $P = 0.001$) (Fig. S1B). As
413 expected due to generally larger cell sizes in microbial eukaryotes compared to prokaryotes,
414 species richness remained higher in the $> 2.7 \mu\text{m}$ vs. 0.2-2.7 μm fractions (Fig. S3C, D). Both
415 the $> 2.7 \mu\text{m}$ and 0.2-2.7 fractions also showed a significant separation between the upper and
416 lower MSR communities (ANOSIM, $> 2.7 \mu\text{m}$: $R = 0.696$, $P = 0.001$; 0.2-2.7 μm : $R = 0.576$, $P =$
417 0.001) (Fig. 2C, D). Overall richness in the $> 2.7 \mu\text{m}$ fraction was also higher in the lower vs.
418 upper river, but this was not true for the 0.2-2.7 μm fraction (Table S1, Richness comparisons).
419 Richness in the $> 2.7 \mu\text{m}$ fraction increased along both the upper and lower river, similarly to
420 prokaryotic communities, but remained relatively stable within the 0.2-2.7 μm fraction.

421 Phosphate was the top environmental factor correlating to the distinction between
422 eukaryotic communities in both filter fractions ($> 2.7 \mu\text{m}$, $R^2 = 0.49$; 0.2-2.7 μm $R^2 = 0.56$)
423 (Table 1). No other factors had correlations > 0.38 (Table 1). At the OTU level, taxa related to an
424 unclassified Ochrophyta (OTU63) and an unclassified Eukaryote (OTU1) separated the 0.2-2.7
425 μm communities ($R^2 > 0.645$, $P = 0.0001$), while the same unclassified Eukaryote OTU (OTU1)
426 and a second unclassified Eukaryote (OTU222) contributed most to separating the $> 2.7 \mu\text{m}$
427 communities ($R^2 > 0.80$, $P = 0.0001$) (Table 2).

428 Stramenopiles (or Heterokonts), encompassing diatoms and many other forms of algae,
429 and OTUs that could not be classified at the phylum level dominated both the $> 2.7 \mu\text{m}$ and 0.2-
430 $2.7 \mu\text{m}$ communities (Fig. 3C, D). Stramenopiles accounted for over 25% of both communities,
431 with higher abundances in the upper vs. lower river. We observed a similar trend of disparate
432 abundances between the upper and lower river for $> 2.7 \mu\text{m}$ Cryptomonadales and 0.2- $2.7 \mu\text{m}$
433 Nucleotmycea, the latter of which include fungi (Fig. 3C; Table S1, 18S OTU table norm.).
434 Within the 0.2- $2.7 \mu\text{m}$ fraction, we identified the five most abundant OTUs as three unclassified
435 *Bacillariophytina* (OTU7, OTU14, OTU9), a *Pythium* sp. (OTU170), and an unclassified
436 *Cryptomonas* (OTU11) (Table S1, 18S OTU table norm.). Comparatively, two unclassified
437 *Eukaryotes* (OTU2 and OTU1), an unclassified *Stramenopiles* (OTU3), an unclassified
438 *Perkinsidae* (OTU13), and an unclassified *Chrysophyceae* (OTU6) had the highest abundance in
439 the $> 2.7 \mu\text{m}$ fraction (Table S1, 18S OTU table norm).

440

441 *The Mississippi River Core Microbiome*

442 We defined the core microbiome as those OTUs detectable after normalization in greater than
443 90% of the sites. The 16S rRNA gene $> 2.7 \mu\text{m}$ and 0.2- $2.7 \mu\text{m}$ core microbiomes consisted of
444 82 and 98 OTUs, respectively, classified into eight different phyla- Proteobacteria,
445 Actinobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Chloroflexi, Chlorobi,
446 Gemmatimonadetes- and composed of taxa such as freshwater SAR11 (LD12, Proteobacteria),
447 *Limnohabitans* sp. (Proteobacteria), *Polynucleobacter* sp. (Proteobacteria), acI clade
448 (Actinobacteria), LD28 clade (Proteobacteria), and *Planktothrix* sp. (Cyanobacteria) (Table S1,
449 16S 0.2- $2.7 \mu\text{m}$ Core Comm. and 16S $> 2.7 \mu\text{m}$ Core Comm.). Core microbome relative
450 abundance in both fractions decreased along the upper river but stabilized in the lower river (Fig.

451 4A). We confirmed this effect by analyzing the upper and lower core microbiomes separately.
452 Although the total OTU numbers changed (81 and 116 OTUs in the upper MSR and 160 and 144
453 OTUs in the lower MSR for the $> 2.7 \mu\text{m}$ and $0.2\text{-}2.7 \mu\text{m}$ fractions, respectively), the trends
454 remained the same (Fig. S5).

455 Eighty OTUs comprised the $> 2.7 \mu\text{m}$ 18S rRNA gene core microbiome (Fig. 4B). We
456 classified these as *Alveolata*, *Cryptophyceae*, *Nucleomycea*, *Stramenopiles*, or unclassified
457 Eukaryota (Table S1, 18S $> 2.7 \mu\text{m}$ Core Comm.). Again, consistent with larger organism sizes,
458 and thus fewer OTUs overall, the $0.2\text{-}2.7 \mu\text{m}$ Eukaryotic core microbiome comprised only 21
459 OTUs (Table S1, 18S $0.2\text{-}2.7 \mu\text{m}$ Core Comm.). These OTUs consisted of *Alveolata*,
460 *Nucleomycea*, *Stramenopiles*, or unclassified Eukaryota (Table S1, 18S $0.2\text{-}2.7 \mu\text{m}$ Core
461 Comm.). While the $0.2\text{-}2.7 \mu\text{m}$ core microbiome remained relatively stable along the river, the $>$
462 $2.7 \mu\text{m}$ core decreased along the upper MSR before stabilizing in the lower river, similarly to
463 that of the prokaryotes (Fig. 4B).

464

465 *Network analyses identify taxa associated with and predictive of eutrophication*

466 We applied Weighted Gene Correlation Network Analysis (WGCNA) to identify co-occurring
467 groups of OTUs (submodules) that also had significant associations with the eutrophication
468 nutrients phosphate and nitrate. Of the submodules identified through WGCNA as being most
469 strongly correlated to phosphate and nitrate, we restrict our discussion to those modeled via PLS
470 analysis to predict $> 50\%$ of the measured nutrient concentration. Our additional PLS analyses
471 for those submodules predicting $< 50\%$ of nitrate and/or phosphate concentrations are included
472 in Figure S6 and Figure S7-S10.

473 Three submodules in the prokaryotic and eukaryotic fractions were strongly associated
474 with phosphate. The 0.2-2.7 μm prokaryotic submodule most associated with phosphate was
475 composed of 51 OTUs (Table 3), and had moderate correlation between the submodule structure
476 and phosphate (Fig. S7D). Strong correlations between submodule structure and the measured
477 nutrient suggests that individual submodule OTUs that also have strong correlations to the
478 nutrient are the most important organisms associated with that nutrient (Langfelder and Horvath
479 2008). PLS modeling determined that this prokaryotic submodule predicted 80% of measured
480 phosphate concentrations (Figure S7E, Table 3). Variable importance in the projection (VIP)
481 analysis found OTUs corresponding to an unclassified *Holophagaceae*, an unclassified
482 *Gemmatimonadaceae*, and an unclassified *Burkholderiaceae* were the three most important in
483 the PLS model for phosphate (Fig. 5A, Table S1, 16S 0.2-2.7 μm PO₄). OTU322 (Acidobacteria
484 subgroup 6), had moderate correlation to phosphate but high node centrality ($n = 51$; Fig. 5A,
485 Table S1, 16S 0.2-2.7 μm PO₄), corroborating evidence that freshwater sediment Acidobacteria
486 subgroup 6 often occur in co-culture with Alphaproteobacteria and may be metabolically
487 connected (Kielak *et al.* 2016 and refs. within). A submodule from the 0.2-2.7 μm eukaryotic
488 fraction was also highly predictive of phosphate (Table 3, Fig. S8D). We identified the top four
489 VIP taxa as an unclassified *Peronosporomycetes*, an unclassified *Ochrophyta*, an unclassified
490 Eukaryote, and an unclassified *Stramenopiles* (Fig. 6A, Table S1, 18S 0.2-2.7 μm PO₄). All four
491 OTUs had Pearson correlations with phosphate greater than 0.60, among which two were
492 negative (Fig. 6A, Table S1, 18S 0.2-2.7 μm PO₄). Unfortunately, the most highly
493 interconnected OTU within the submodule remained unclassified even at the Phylum level (Fig.
494 6A, Table S1, 18S 0.2-2.7 μm PO₄). A $> 2.7 \mu\text{m}$ eukaryotic submodule could predict 62% of

495 measured phosphate variance (Table 3). An unclassified Eukaryote and an unclassified
496 *Peronosporomycetes* occupied top two VIP positions (Fig. 6B, Table S1, 16S > 2.7 μm PO₄).
497 Only two submodules had strong associations with nitrate. The > 2.7 μm prokaryotic
498 submodule most strongly correlated with nitrate contained 133 OTUs (Table 3, Fig. S9A).
499 However, despite a low structural correlation to nitrate, the submodule strongly predicted
500 measured nitrate in the PLS model (Figure S9B, Table 3). The four highest VIP scoring OTUs
501 were an *Anabaena* sp., a *Flavobacterium* sp., an unclassified bacterium, and a member of the
502 *Sphingobacteriales* NS11-12 marine group (Fig. 5B, Table S1, 16S > 2.7 μm NO₃). OTUs with
503 the highest node centrality (> 20) belonged to the *Sphingomonadales* (*Alphaproteobacteria*) and
504 *Sphingobacteriales* (Bacteroidetes), and each positively correlated with nitrate ($R > 0.48$, Fig.
505 5B, Table S1, 16S > 2.7 μm NO₃). The > 2.7 μm eukaryotic submodule most associated with
506 nitrate predicted 57% of observed variation in nitrate concentrations (Table 3). OTUs with the
507 top VIP scores were two unclassified *Chrysophyceae*, an unclassified *Ochrophyta*, and an
508 unclassified Diatom (Fig. 6C, S10; Table S1, 18S > 2.7 μm NO₃).

509

510 **Discussion**

511 Understanding microbial communities on a whole-river scale is essential for linking the complex
512 relationships between microorganisms, metabolism, and water quality. At 2914 km, this study is
513 the largest transect of the MSR to date, and the first to include both prokaryotic and eukaryotic
514 data by size fractions. This is also the largest river yet surveyed at this level of geographic
515 completion, which may account for some of its distinctive microbiological features. Although the
516 MSR comprised similar aquatic microbial taxa found within other rivers (Zwart *et al.* 2002;
517 Cottrell *et al.* 2005; Ghai *et al.* 2011; Fortunato *et al.* 2013; Jackson *et al.* 2014; Read *et al.* 2015;

518 Savio *et al.* 2015; Meziti *et al.* 2016), it hosted unique relative abundance and core microbiome
519 trends, indicating that rivers have distinguishing microbiome characteristics. Furthermore, we
520 observed two different community regimes separated at the Missouri River confluence and
521 mirrored by the measured physio-chemical properties of the MSR (Fig. 1B). This is an important
522 hydrological feature of the MSR that strongly influences its microbiology, and further
523 distinguishes it from other rivers. Our study also identified taxa associated with, and predictive
524 of, the eutrophication nutrients nitrate and phosphate that provide important targets for future
525 study and may assist in detecting and quantifying imminent changes in river water quality.

526 MSR microbial communities separated into two distinct fractions of microbial
527 assemblages, 0.2-2.7 μm and $> 2.7 \mu\text{m}$ (ANOSIM, $R = 0.65$, $P = 0.001$), throughout the river
528 transect, a common feature of aquatic ecosystems (DeLong *et al.* 1993; Crump *et al.* 1999;
529 Allgaier and Grossart 2006; D'Ambrosio *et al.* 2014; Jackson *et al.* 2014). However, despite
530 sharing similar taxa with other rivers (Ghai *et al.* 2011; Fortunato *et al.* 2012; Crump *et al.* 2012;
531 Read *et al.* 2015; Savio *et al.* 2015; Meziti *et al.* 2016; Niño-García *et al.* 2016), MSR microbial
532 community abundances were distinct at a phylum level from previous work on Thames River
533 (Read *et al.* 2015), Danube River (Savio *et al.* 2015), Canadian Boreal Rivers (Niño-García *et al.*
534 2016), Toolik catchment (Crump *et al.* 2012), and Yenisei River (Kolmakova *et al.* 2014).
535 Specifically, within the 0.2-2.7 μm MSR prokaryotic fraction, Proteobacteria remained the most
536 abundant phylum throughout the river, while Bacteroidetes and Actinobacteria decreased
537 downriver (Fig. 3). In contrast, other river studies found headwaters dominated by Bacteroidetes
538 that gave way to more abundant Actinobacteria and Proteobacteria further downriver (Read *et al.*
539 2015; Savio *et al.* 2015). Similarly, the trends observed in the upper portion of the MSR (Staley
540 *et al.* 2013), where Bacteroidetes and Actinobacteria decreased while Proteobacteria increased,

541 diverged from the trends found in this study (Fig. 3). Further, while no clear increasing or
542 decreasing trends were observed in Payne *et al.* (2017), Actinobacteria occurred with the highest
543 relative abundances in the planktonic fraction, whereas in this study, Proteobacteria were the
544 most abundant phylum. This may be attributable to the difference in sampling year or possibly
545 methodology, such as an amplification step that was not undertaken in this work (Payne *et al.*
546 2017). Furthermore, although many of the dominant taxa found in other rivers also occupied the
547 MSR (LD12 (freshwater SAR11, *Alphaproteobacteria*), *Limnohabitans* sp.
548 (*Betaproteobacteria*), LD28 (*Betaproteobacteria*), acI clade (Actinobacteria), and *Algoriphagus*
549 sp. (Bacteroidetes) (Ghai *et al.* 2011; Fortunato *et al.* 2013; Kolmakova *et al.* 2014; Read *et al.*
550 2015; Savio *et al.* 2015; Meziti *et al.* 2016), we observed OTU-level abundance differences at a
551 whole-river scale. For example, OTUs belonging to the acI and LD12 freshwater clades
552 continually increased in abundance towards the river mouth in the Thames and Danube Rivers
553 (Read *et al.* 2015; Savio *et al.* 2015), whereas MSR OTUs classified as acI (OTUs 3, 4 and 7)
554 and LD12 (OTU 11) did not show any distinct change in abundance throughout the transect.
555 Specific to studies of portions of the MSR,

556 The generally greater richness (Table S1, Richness comparisons) and decreased core
557 microbial community abundances (Fig. 4) in the lower vs. upper MSR contrasted predictions by
558 the RCC (Vannote *et al.*, 1980) and observations in previous studies from both large and small
559 rivers (Read *et al.* 2015; Savio *et al.* 2015, Niño-García *et al.* 2016) where these trends were
560 reversed. Importantly, we did not sample the true headwaters of the MSR (Lake Itasca to above
561 St. Cloud), and at the point of first sampling, the MSR already constituted an eighth order river.
562 Therefore, some of the trends predicted by the RCC may have been missed, although increasing
563 richness was also observed in the lower MSR during a different year (Payne *et al.* 2017).

564 Ultimately, the variant observations between this MSR survey and other river studies may result
565 from different sampling methodologies or the particular timing of sampling. Previous studies
566 conducted on six arctic rivers (Crump *et al.* 2009), two smaller temperate rivers (Parker and
567 Ipswich Rivers) (Crump and Hobbie 2005), and the upper portion of the MSR (Staley *et al.*
568 2015) showed that seasonality was important in structuring bacterial communities over three year
569 and two year sampling periods.

570 However, our observed differences may also stem from biological signal related to
571 unique environmental conditions, human impacts (Meziti *et al.* 2016; Payne *et al.* 2017), and
572 changes in hydrology and the level of river engineering (Ruiz-González *et al.* 2013, 2015;
573 Freimann *et al.* 2015; Niño-García *et al.* 2016) with distance. For instance, the most striking
574 difference between the MSR and other rivers was the distinct separation of prokaryotic and
575 eukaryotic community regimes at the Missouri River confluence. This separation matched
576 physio-chemical changes observed for the MSR such as increased Strahler river order (8 to 10)
577 and more consistent nutrient concentrations in the lower river compared to the upper (Fig. 1B)
578 (Pierson *et al.* 2008). These results are bolstered by another observation depicting a major
579 separation of communities above and below the Missouri confluence, observed during a different
580 year (2012) and roughly three months prior (late summer) to the current study (Payne *et al.*
581 2017). This similarity to our results suggests the major influence of the Missouri River
582 confluence is not seasonally dependent.

583 Although the overall microbial community patterns in the MSR differed from other
584 rivers, we hypothesize that similar ecological processes drove the separation between the upper
585 and lower MSR communities, namely changes in the importance of immigration, emigration, and
586 resource gradient dynamics (Crump *et al.* 2012; Savio *et al.* 2015; Niño-García *et al.* 2016).

587 Specifically, our data suggest that mass effects play a role in structuring microbial communities
588 in the upper MSR, although instead of only in the headwaters, this process continues for almost a
589 third of the length of the river, possibly due to contributions from large tributaries and abundant
590 dams. This contrasts with the conclusion made by Staley *et al.* (2015) for a smaller transect in
591 the upper MSR, where they suggested species sorting was the primary influence structuring
592 upper MSR microbial communities. Increased turbidity correlated with decreased core
593 microbiome relative abundance (Spearman rank correlation, $> 2.7 \mu\text{m}$ $R = 0.53$; $0.2\text{-}2.7 \mu\text{m}$ $R =$
594 0.63) in the upper MSR, and nutrients like phosphate and nitrate continually increased along the
595 upper river in the current study (Fig. 1B). Similarly, richness also increased along the upper river
596 (except in the $0.2\text{-}2.7 \mu\text{m}$ 18S communities) (Fig. S3). These patterns are consistent with
597 communities under the influence of strong mass effects in the upper MSR, whereby inputs from
598 allochthonous sources continually contributed nutrients, particulate matter, and additional
599 microbial taxa.

600 Conversely, nitrate, phosphate, turbidity, and core microbiome relative abundance all
601 stabilized in the lower MSR (Figs. 1B, 4), suggesting a change in the relative influence of
602 external sources for these variables. We speculate that once the MSR grew to a tenth order river,
603 its large volume and size buffered it from allochthonous inputs. Nevertheless, overall richness
604 was greater in the lower river compared to the upper (except in the $0.2\text{-}2.7 \mu\text{m}$ 18S communities)
605 (Table S1, Richness comparisons), and, like the upper river, richness generally increased in the
606 lower river (Fig. S3), matching a previous observation (Payne *et al.* 2017). However, since the
607 relative abundance of core microbiome taxa remained comparatively stable in the lower river
608 (Fig. 4), increased richness likely occurred primarily from the emergence of low abundance
609 OTUs. The extent to which a local or regional event impacts downriver populations is dependent

610 on the success of allochthonous taxa associated with such an event to become established within
611 the autochthonous population (Crump *et al.* 2012; Niño-García *et al.* 2016). The larger the river,
612 the less likely any newly immigrated taxa will establish dominance. Thus, a plausible scenario
613 for the regime change observed at the Missouri River confluence is that the influence of mass
614 effects was drastically reduced by the increased size of the lower river, and that allochthonous
615 taxa immigrating there were relegated to lower abundances.

616 Alternatively, some of the increase in richness may have occurred through native
617 community differentiation within the lower river. An overall community shift from a mixture of
618 allochthonous members to a “native” population requires growth rates greater than residence
619 time over a given distance (Crump *et al.* 2004). Though river speed increased along the MSR,
620 the effective residence time also increased since taxa no longer experienced rapidly changing
621 environmental variables (Fig. 1B). As a result, the lower river may have provided opportunities
622 for microbial community differentiation based on microniches within the river, which is possible
623 considering average prokaryotic growth rates (Savio *et al.* 2015), especially among particle-
624 associated ($> 2.7 \mu\text{m}$) taxa (Crump *et al.* 1998). Thus, although the aggregate patterns in
625 particular phyla and the overall taxonomic richness within the MSR differ from other systems,
626 similar ecological processes may still drive these patterns, but the relative proportion of the river
627 whereby mass effects vs. species sorting dominates fosters unique community assemblages
628 (Niño-García *et al.* 2016). To resolve the relative importance of mass effects vs. species sorting
629 in the upper and lower rivers, future work will need to incorporate explicit measurements of
630 immigrating and emigrating taxa, as well as growth rates for different organisms at different
631 geographic positions.

632 Within this dynamic river system, we also sought to define the microorganisms most
633 associated with eutrophication nutrients to provide specific taxa for future study of nitrate and
634 phosphate uptake and/or metabolism, and also as plausible biological indicators of river trophic
635 state. By focusing on OTUs with the highest VIP scores in the PLS models, we identified several
636 bacterial and eukaryotic taxa that fit these criteria (Figs. 5, 6). Organisms in the
637 *Sphingomonadaceae* (e.g., *Novosphingobium* spp.) contributed strongly to the PLS models
638 predicting nitrate with both $> 2.7 \mu\text{m}$ (Fig. 5B) and $0.2\text{-}2.7 \mu\text{m}$ (Fig. S6A) size fractions (Table
639 S1, 16S $0.2\text{-}2.7 \mu\text{m}$ VIP NO₃ and 16S $> 2.7 \mu\text{m}$ VIP NO₃). *Novosphingobium* spp. isolates have
640 previously been associated with eutrophic environments (Trusova and Gladyshev 2002; Zwart *et*
641 *al.* 2002; Addison *et al.* 2007; Li *et al.* 2012) and some can reduce nitrate (Addison *et al.* 2007;
642 Li *et al.* 2012), making these specific OTUs candidates for nitrate metabolism in the river water
643 column. An *Anabaena* sp. (OTU40) from the core microbiome (Table S1, 16S $> 2.7 \mu\text{m}$ Core
644 Comm.) had the top VIP score within the $> 2.7 \mu\text{m}$ submodule that could predict 69% of nitrate
645 concentrations, and correlated negatively with nitrate (Table 3, Fig. 5B). The nitrogen-fixing
646 *Anabaena* spp. (Allen and Arnon 1955) typically bloom in low dissolved inorganic nitrogen
647 (DIN) conditions (Wood *et al.* 2010), making the absence of these consistent with high DIN. An
648 unclassified *Holophagaceae* OTU (OTU33) had the highest VIP score within the prokaryotic
649 $0.2\text{-}2.7 \mu\text{m}$ submodule that predicted 80% of the variance in phosphate concentrations (Table 3,
650 Fig. 5A). This same OTU was also a key driver of the beta diversity separation between upper
651 and lower river communities (Table 2), and a member of the core microbiome (Table S1, 16S
652 $0.2\text{-}2.7 \mu\text{m}$ Core Comm.). The *Holophagaceae* belong to the Acidobacteria phylum, and the
653 Ohio River contained a much higher abundance of Acidobacteria relative to other tributaries in a
654 previous study (Jackson *et al.* 2014). Notably, Acidobacteria increased with river distance in our

655 study as well (Fig. S4), with a peak in the 0.2-2.7 μm fraction near the Arkansas River
656 confluence. This increase in “free-living” Acidobacteria downriver is distinct from other whole
657 river studies, making these organisms, and the *Holophagaceae* OTUs in particular, potentially
658 important organisms for the MSR river basin specifically.

659 Within Eukaryotes, multiple different algae, diatom, and Oomycetes OTUs occupied
660 submodules highly predictive of nitrate and phosphate (Fig. 6A-C), and specifically
661 *Chrysophyceae* taxa from both size fractions correlated strongly with nitrate (Fig. 6C, Fig. S6E).
662 *Chrysophyceae* (golden algae) commonly occupy river systems (Necchi Jr 2016) including the
663 MSR (Korajkic *et al.* 2015), can be autotrophic and mixotrophic (Jansson *et al.* 1996), and may
664 serve as predators of prokaryotes (Caron *et al.* 1990). Further, multiple OTUs classified as
665 *Peronosporomycetes* were important in predicting phosphate in both size fractions (Fig 6A, B,
666 Table S1, 18S 0.2-2.7 μm VIP PO₄ and 18S > 2.7 μm VIP PO₄). *Peronosporomycetes* are
667 fungus-like eukaryotic organisms known to be pathogenic in fish, plants, and mammals (Dick
668 2003; Islam and von Tiedemann 2011). While we also identified many other eukaryotic OTUs as
669 important predictors of nutrients, poor taxonomic resolution hindered our ability to discuss them
670 further. Improved cultivation and systematics of key microbial eukaryotes will be vital to
671 understanding river nutrient dynamics.

672 While the most geographically comprehensive analysis to date for the MSR, this study
673 only encompasses a snapshot in time. As noted previously, seasonal changes that have been
674 observed in other rivers (Crump and Hobbie 2005; Crump *et al.* 2009; Smith *et al.* 2010;
675 Fortunato *et al.* 2013; Staley *et al.* 2015) undoubtedly influence this dynamic system, though the
676 permanence of the regime change at the Missouri River confluence remains in question. Future
677 studies should incorporate microbial responses, at a whole-river scale, to seasonal pulse events

678 (e.g. rain, snow melt), and how river size and volume may buffer local microbial communities
679 from allochthonous inputs (Zeglin 2015). Our current research highlights the distinctiveness of
680 MSR microbial communities and the complexities influencing their structure within the MSR
681 ecosystem. The observed association between changes in Strahler's river order, nutrient
682 dynamics, and community composition indicates the importance of hydrology on the spatial
683 dynamics structuring microbial communities (Freimann *et al.* 2015; Zeglin 2015; Niño-García *et*
684 *al.* 2016) and provides baseline information for future MSR studies that incorporate greater
685 temporal and spatial resolution. With river water quality of growing local and global importance
686 (Vorosmarty *et al.* 2010; Russell and Weller 2013), the candidate taxa predictive of eutrophic
687 nutrients determined herein also provide important targets for further research into their roles for
688 indicating, and potentially improving, river health.

689

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705

706 **Conflict of Interest**

707 The authors declare no competing financial interests.

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Tables

Table 1. Summary of the correlations between environmental variables and microbial community beta diversity ordination on NMDS plots in Figure 2. +/- designates the direction of the association between the environmental variable and NMDS axis

Variable		NMDS1	NMDS2	P-value	R ²		NMDS1	NMDS2	P-value	R ²
<i>PO₄³⁻</i>	16S rRNA Gene 0.2-2.7 μm	+	+	0.001	0.404	16S rRNA Gene > 2.7 μm	+	+	0.001	0.575
<i>SiSO₄</i>		-	+	0.003	0.183		-	-	0.015	0.115
<i>NO₃⁻</i>		+	+	0.001	0.443		+	+	0.001	0.33
<i>NH₄⁺</i>		-	-	0.002	0.178		-	-	0.004	0.189
<i>NO₂⁻</i>		Not significant					Not significant			
<i>River Speed</i>		-	-	0.001	0.261		-	-	0.001	0.39
<i>Water Temperature</i>		-	-	0.001	0.385		-	-	0.001	0.524
<i>Turbidity</i>		-	-	0.001	0.409		-	-	0.001	0.536
<i>PO₄³⁻</i>	18S rRNA Gene 0.2-2.7 μm	+	+	0.001	0.555	18S rRNA Gene > 2.7 μm	+	-	0.001	0.486
<i>SiSO₄</i>		-	+	0.001	0.264		-	-	0.001	0.265
<i>NO₃⁻</i>		Not significant					+	+	0.001	0.2
<i>NH₄⁺</i>		-	+	0.019	0.106		Not significant			
<i>NO₂⁻</i>		Not significant					Not significant			
<i>River Speed</i>		-	-	0.001	0.282		-	-	0.001	0.345
<i>Water Temperature</i>		-	-	0.001	0.29		-	-	0.001	0.372
<i>Turbidity</i>		-	-	0.001	0.272		-	-	0.001	0.353

Table 2. Summary of the correlations between OTUs and microbial community beta diversity ordination on NMDS plots in Figure 2. +/- designates the direction of the association between the OTU and NMDS axis.

	OTU	Classification	NMDS1	NMDS2	P-value	R ²		OTU	Classification	NMDS1	NMDS2	P-value	R ²
16S rRNA Gene 0.2-2.7 μm	<i>OTU372</i>	Unc. Bacteria	+	-	0.0001	0.604	16S rRNA Gene > 2.7 μm	<i>OTU3</i>	acI clade	+	+	0.0001	0.796
	<i>OTU135</i>	<i>Flavobacterium</i> sp.	-	-	0.0001	0.522		<i>OTU181</i>	Unc. Bacillaceae	-	+	0.0001	0.773
	<i>OTU29</i>	<i>Fluviicola</i> sp. Unc.	-	-	0.0001	0.509		<i>OTU145</i>	<i>Desulfosporosinus</i> sp.	-	+	0.0001	0.742
	<i>OTU48</i>	Microbacteriaceae	-	+	0.0001	0.508		<i>OTU13</i>	Unc. Bacillales Unc.	-	+	0.0001	0.740
	<i>OTU115</i>	<i>Flavobacterium</i> sp.	-	+	0.0001	0.503		<i>OTU68</i>	Micromonosporaceae	-	+	0.0001	0.726
	<i>OTU33</i>	Unc. Holophagaceae	+	+	0.0001	0.501		<i>OTU202</i>	Unc. Bacillales Unc.	-	+	0.0001	0.725
	<i>OTU161</i>	<i>Flavobacterium</i> sp.	-	-	0.0001	0.501		<i>OTU60</i>	Gemmatimonadaceae	+	+	0.0001	0.716
	<i>OTU100</i>	<i>Clostridium</i> sp. Unc.	+	+	0.0001	0.497		<i>OTU89</i>	Unc. Bacillales Unc.	-	+	0.0001	0.702
	<i>OTU85</i>	Peptostreptococcaceae Unc.	+	+	0.0001	0.496		<i>OTU169</i>	Micromonosporaceae	-	+	0.0001	0.698
	<i>OTU62</i>	Comamonadaceae	-	+	0.0001	0.493		<i>OTU28</i>	Unc. Planococcaceae	-	+	0.0001	0.694
18S rRNA Gene 0.2-2.7 μm	<i>OTU1</i>	Unc. Eukaryota	+	+	0.0001	0.693	18S rRNA Gene > 2.7 μm	<i>OTU1</i>	Unc. Eukaryote	+	+	0.0001	0.870
	<i>OTU63</i>	Unc. Ochrophyta	-	-	0.0001	0.645		<i>OTU222</i>	Unc. Ochrophyta	+	+	0.0001	0.806
	<i>OTU241</i>	Unc. Ochrophyta	-	+	0.0001	0.642		<i>OTU3</i>	Unc. Stramenopiles	+	-	0.0001	0.796
	<i>OTU133</i>	Unc. Ochrophyta	-	+	0.0001	0.632		<i>OTU1984</i>	Unc. Eukaryote	+	-	0.0001	0.794
	<i>OTU219</i>	Unc. Eukaryota	+	+	0.0001	0.630		<i>OTU345</i>	Unc. Ochrophyta Unc.	-	-	0.0001	0.792
	<i>OTU113</i>	Unc. Alveolata	+	+	0.0001	0.616		<i>OTU112</i>	Cryptomonadales	+	+	0.0001	0.779
	<i>OTU154</i>	Unc. Ochrophyta	-	-	0.0001	0.598		<i>OTU63</i>	Unc. Eukaryote Chrysophyceae clone	+	+	0.0001	0.773
	<i>OTU481</i>	Unc. Eukaryota	-	-	0.0001	0.598		<i>OTU154</i>	P34.45	-	-	0.0001	0.773
	<i>OTU457</i>	Unc. Eukaryota	-	-	0.0001	0.596		<i>OTU155</i>	Bacillariophytina	+	+	0.0001	0.765
	<i>OTU3</i>	Unc. Stramenopile	+	+	0.0001	0.594		<i>OTU1146</i>	Unc. Eukaryote	+	+	0.0001	0.765

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Table 3. Correlations between prokaryotic and eukaryotic submodules to nitrate and phosphate, as well as PLS model results

<i>Fraction</i>	<i>Gene</i>	<i>Nutrient</i>	<i>Submodule</i>	<i>Eigen Correlations</i>	<i>Total OTUs</i>	<i>PLS model</i>
0.2-2.7 μm	16S	NO_3^{2-}	Blue	R = 0.6, P = 7e-08	77	$R^2=0.35$; R = 0.65, P = 1.e-09
> 2.7 μm	16S	NO_3^{2-}	Brown	R = 0.56, P = 3e-07	133	$R^2 = 0.69$; R = 0.83, P = < 2.2e-16
0.2-2.7 μm	16S	PO_4^{3-}	Turquoise	R = 0.53, P = 2e-06	51	$R^2=0.80$; R = 0.89, P = < 2.3e-16
> 2.7 μm	16S	PO_4^{3-}	Yellow	R = 0.53, P = 1e-06	80	$R^2 = 0.48$; R = 0.77, P = 4.88e-15
0.2-2.7 μm	18S	NO_3^{2-}	Green	R = 0.39, P = 6e-04	39	$R^2=0.38$; R = 0.62, P = 2.97e-9
> 2.7 μm	18S	NO_3^{2-}	Brown	R = 0.52, P = 2e-06	59	$R^2=0.572$; R = 0.759, P = 6.7e-15
0.2-2.7 μm	18S	PO_4^{3-}	Turquoise	R = 0.56, P = 2e-07	56	$R^2=0.80$; R = 0.89, P = < 2e-16
> 2.7 μm	18S	PO_4^{3-}	Red	R = 0.60, P = 2e-08	39	$R^2 = 0.618$; R = 0.799, P = < 2e-16

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1004

1005 **Figure Legends**

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1007 **Figure 1.** Sampling map (A) with graph inserts that represent the measured discharge rate (cubic
1008 meter second⁻¹ [cms]) as recorded on the USGS gauges, and six environmental parameters
1009 measured along the transect, according to concentration or visible depth of secchi disk by
1010 distance (B). Throughout the figure and text, blue and red dots represent sampling
1011 locations above and below the Missouri River confluence, respectively, and are designated
1012 throughout as “upper” and “lower.” Cell Counts only represent the Planktonic (< 2.7 μm)
1013 fraction.

1014 **Figure 2.** Non-metric multidimensional scaling (NMDS) results for whole community. The four
1015 plots represent > 2.7 μm (A, C) and 0.2-2.7 μm (B, D) fractions for the 16S (A, B) and
1016 18S (C, D) rRNA gene communities.

1017 **Figure 3.** Relative abundance, according to transect distance, for the top 6 and 5 phyla in the 16S
1018 rRNA gene > 2.7 μm (A) and 0.2-2.7 μm (B) communities and 18S rRNA gene > 2.7 μm
1019 (C) and 0.2-2.7 μm (D) communities, respectively. Non-linear regressions with 95% CI
1020 (gray shading) are provided for reference.

1021 **Figure 4.** Core microbiome aggregate abundance for the 16S (A) and 18S (B) rRNA gene. In
1022 each, triangles and circles points represent 0.2-2.7 μm and > 2.7 μm fractions,
1023 respectively. Non-linear regressions with 95% confidence intervals (CI) (gray shading) are
1024 provided for reference.

1025 **Figure 5.** PLS results for the 16S rRNA gene community submodules most associated with
1026 phosphate (A) and nitrate (B). OTU correlation with a given nutrient is indicated on the y-
1027 axis according to the number of co-correlations (node centrality) on the x-axis.
1028 Community fractions: 0.2-2.7 μm (A) and > 2.7 μm (B). Circle size is proportional to VIP

1029 scores, with top 10 VIP scoring and top node centrality OTUs labeled with their highest-
1030 resolution taxonomic classification and OTU number. Colors represent the taxonomic
1031 classification the phylum level. For all OTU taxonomic designations resulting from this
1032 analysis, see Table S1 “16S 0.2-2.7 μ m PO₄” and “16S > 2.7 μ m NO₃”.

1033 **Figure 6.** PLS results for the 18S rRNA gene community submodules most associated with
1034 phosphate and nitrate. OTU correlation with a given nutrient is indicated on the y-axis
1035 according to the number of co-correlations (node centrality) on the x-axis. Community
1036 fractions: 0.2-2.7 μ m (A) and > 2.7 μ m (B, C). Circle size is proportional to VIP scores,
1037 with top 10 VIP scoring and top node centrality OTUs labeled with their highest-
1038 resolution taxonomic classification and OTU number. Colors represent the taxonomic
1039 classification the phylum level. For all OTU taxonomic designations resulting from this
1040 analysis, see Table S1 “18S 0.2-2.7 μ m NO₃”, “16S > 2.7 μ m PO₄”, and “16S >2.7 μ m
1041 NO₃”

1042

1043 **Supplemental Figure Legends**

1044

1045 **Figure S1.** NMDS results for the 16S (A) and 18S (B) rRNA gene communities. In each, circles
1046 and triangles represent the > 2.7 μ m and 0.2-2.7 μ m fractions, respectively.

1047 **Figure S2.** Hclust results for the 16S (A) and 18S (B) rRNA gene communities.

1048 **Figure S3.** Separated upper and lower MSR Richness and Evenness indexes for the 16S > 2.7
1049 μ m (A) and 0.2-2.7 μ m (B) and 18S > 2.7 (C) and 0.2-2.7 μ m (D) communities. Linear
1050 regressions with 95% CI (gray shading) are provided for reference.

1051 **Figure S4.** Relative abundance, by phylum, according to transect distance, for phyla accounting

1052 for > 0.1% of the total reads for the 16S rRNA (A, B) and 18S rRNA genes (C, D) > 2.7
1053 μm (A, C) and 0.2-2.7 μm (B, D) communities. Non-linear regressions with 95% CI (gray
1054 shading) are provided for reference.

1055 **Figure S5.** Separated upper and lower MSR core microbiome aggregate abundance for the 16S
1056 rRNA gene communities. For the upper and lower river, the core microbiome was defined
1057 separately requiring OTUs to have greater than one read in 90% of the samples. > 2.7 μm
1058 (A) and 0.2-2.7 μm (B) 16S rRNA gene communities in the upper and lower MSR.

1059 **Figure S6.** PLS results for the 0.2-2.7 μm and > 2.7 μm 16S (A-D) and 18S (E-H) rRNA gene
1060 community for selected submodules with nitrate and phosphate and a VIP score > 1.
1061 Correlation of submodule OTUs to nitrate and phosphate according to the number of co-
1062 correlations (node centrality) for 0.2-2.7 μm (A, B, E, F) and > 2.7 μm (C, D, G, H) 16S
1063 and 18S rRNA gene communities. Circle size is proportional to VIP scores, with top 10
1064 VIP scoring and top node centrality OTUs labeled with their highest-resolution taxonomic
1065 classification and OTU number. Colors represent the taxonomic classification the phylum
1066 level.

1067 **Figure S7.** WCGNA results for 0.2-2.7 μm 16S rRNA gene community submodules of interest
1068 based on Pearson correlations to environmental measurements (A). Color shading depicts
1069 the strength of the Pearson correlation with individual submodule's eigenvalue. Boxes
1070 indicate the selected submodule with its Pearson correlation and P-value. Graphs of the
1071 relationship between the selected submodule OTUs and the strength of the individual
1072 OTUs to nitrate (B) and phosphate (D). PLS regression of the predicted nutrient
1073 concentrations versus measured nutrient concentrations (C, E) using the corresponding
1074 selected submodules. Linear regressions with 95% CI (gray shading) are provided for

1075 reference.

1076 **Figure S8.** WCGNA results for 0.2-2.7 μm 18S rRNA gene community submodules of interest
1077 based on Pearson correlations to environmental measurements (A). Color shading depicts
1078 the strength of the Pearson correlation with individual submodule's eigenvalue. Boxes
1079 indicate the selected submodule with its Pearson correlation and P-value. Graphs of the
1080 relationship between the selected submodule OTUs and the strength of the individual
1081 OTUs to nitrate (B) and phosphate (D). PLS regression of the predicted nutrient
1082 concentrations versus measured nutrient concentrations (C, E) using the corresponding
1083 selected submodules. Linear regressions with 95% CI (gray shading) are provided for
1084 reference.

1085 **Figure S9.** WCGNA results for $> 2.7 \mu\text{m}$ 16S rRNA gene community submodules of interest
1086 based on Pearson correlations to environmental measurements (A). Color shading depicts
1087 the strength of the Pearson correlation with individual submodule's eigenvalue. Boxes
1088 indicate the selected submodule with its Pearson correlation and P-value. Graphs of the
1089 relationship between the selected submodule OTUs and the strength of the individual
1090 OTUs to nitrate (B) and phosphate (D). PLS regression of the predicted nutrient
1091 concentrations versus measured nutrient concentrations (C, E) using the corresponding
1092 selected submodules. Linear regressions with 95% CI (gray shading) are provided for
1093 reference.

1094 **Figure S10.** WCGNA results for $> 2.7 \mu\text{m}$ 18S rRNA gene community submodules of interest
1095 based on Pearson correlations to environmental measurements (A). Color shading depicts
1096 the strength of the Pearson correlation with individual submodule's eigenvalue. Boxes
1097 indicate the selected submodule with its Pearson correlation and P-value. Graphs of the

1098 relationship between the selected submodule OTUs and the strength of the individual
1099 OTUs to nitrate (B) and phosphate (D). PLS regression of the predicted nutrient
1100 concentrations versus measured nutrient concentrations (C, E) using the corresponding
1101 selected submodules. Linear regressions with 95% CI (gray shading) are provided for
1102 reference.

1103

1104 **Supporting Tables**

1105 Supplemental Table S1 is a spreadsheet, TableS1.xlsx. Tabs include General Information,
1106 Spearman Rank, 0.2-2.7 μm and $> 2.7 \mu\text{m}$ 16S and 18S rRNA gene Core communities, 0.2-2.7
1107 μm and $> 2.7 \mu\text{m}$ 16S and 18S rRNA WGCNA, and 16S and 18S rRNA gene OTU tables
1108 (trimmed and normalized).

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1110 Additional Supplemental Information, including R scripts, Table S1, and our Mothur workflow
1111 are hosted on the Thrash Lab website at: thethrashlab.com/publications.

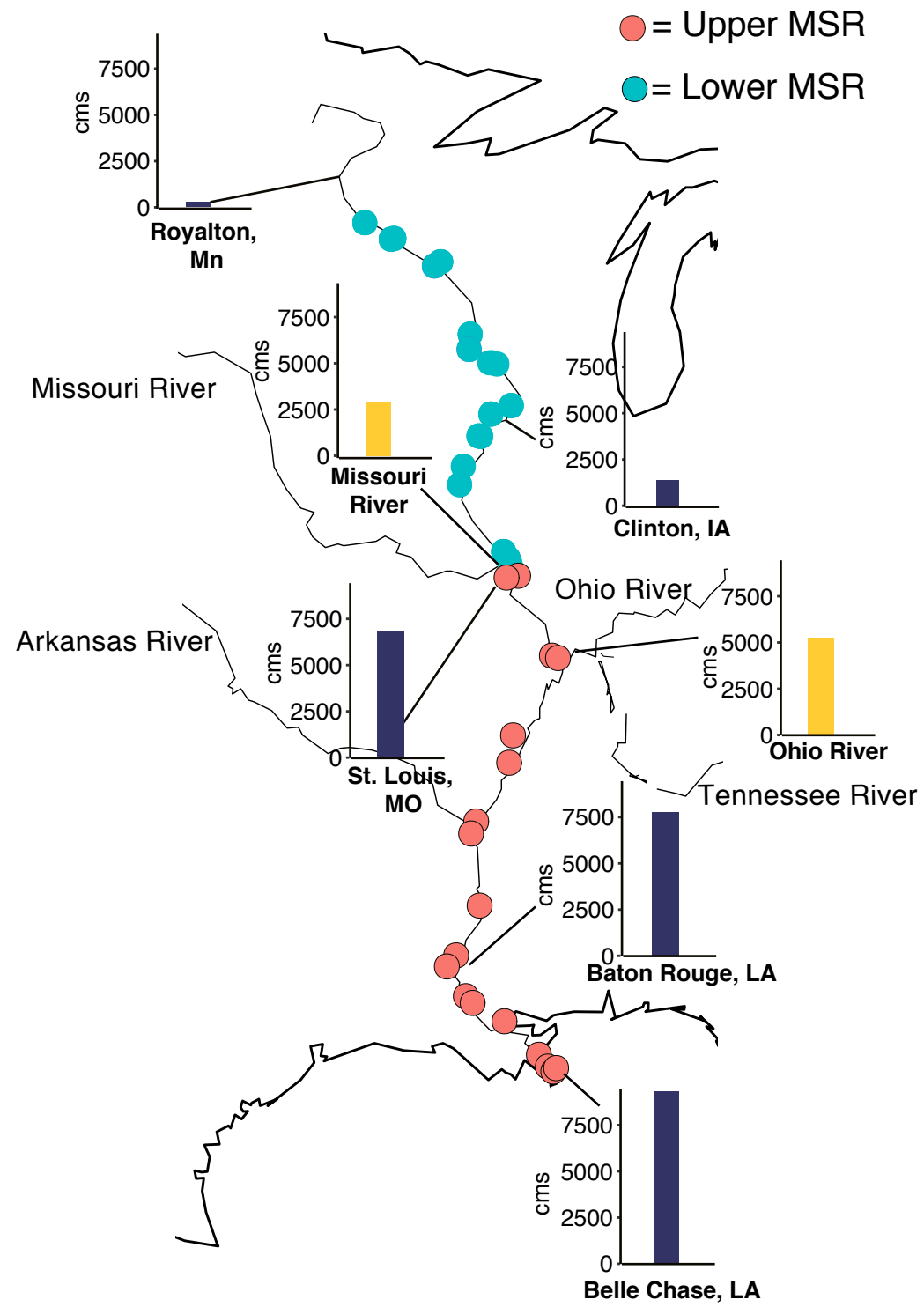
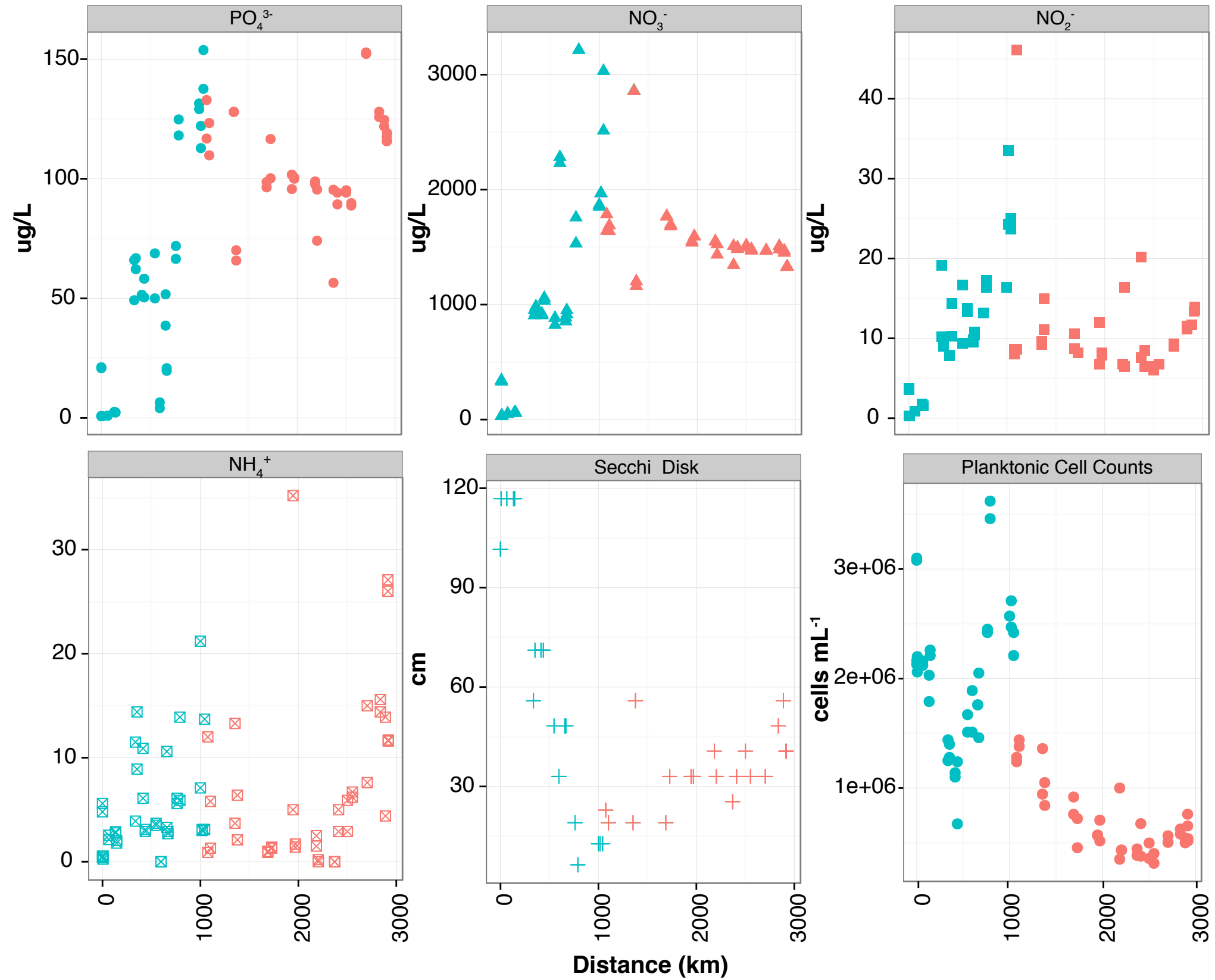
Figure 1A**B**

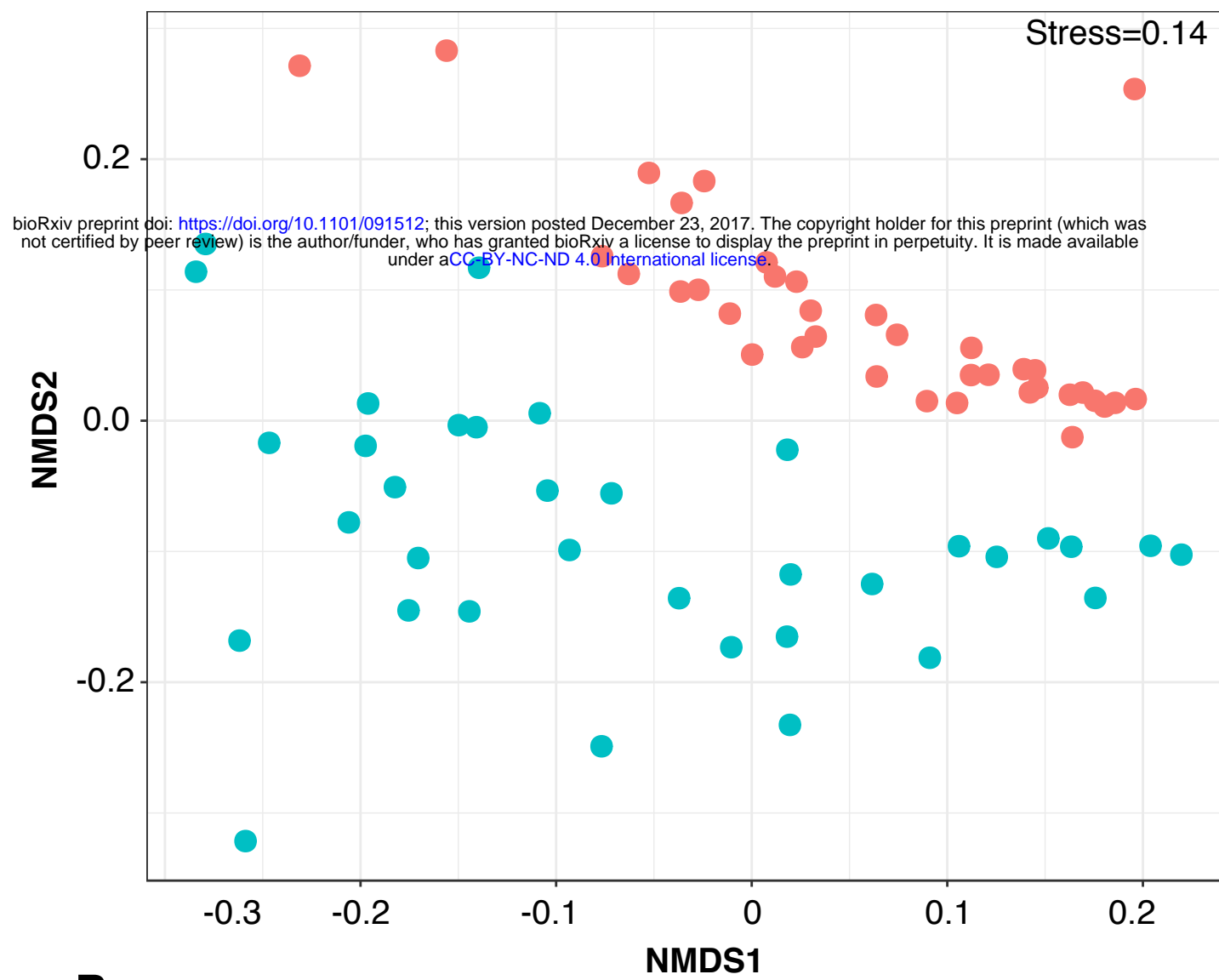
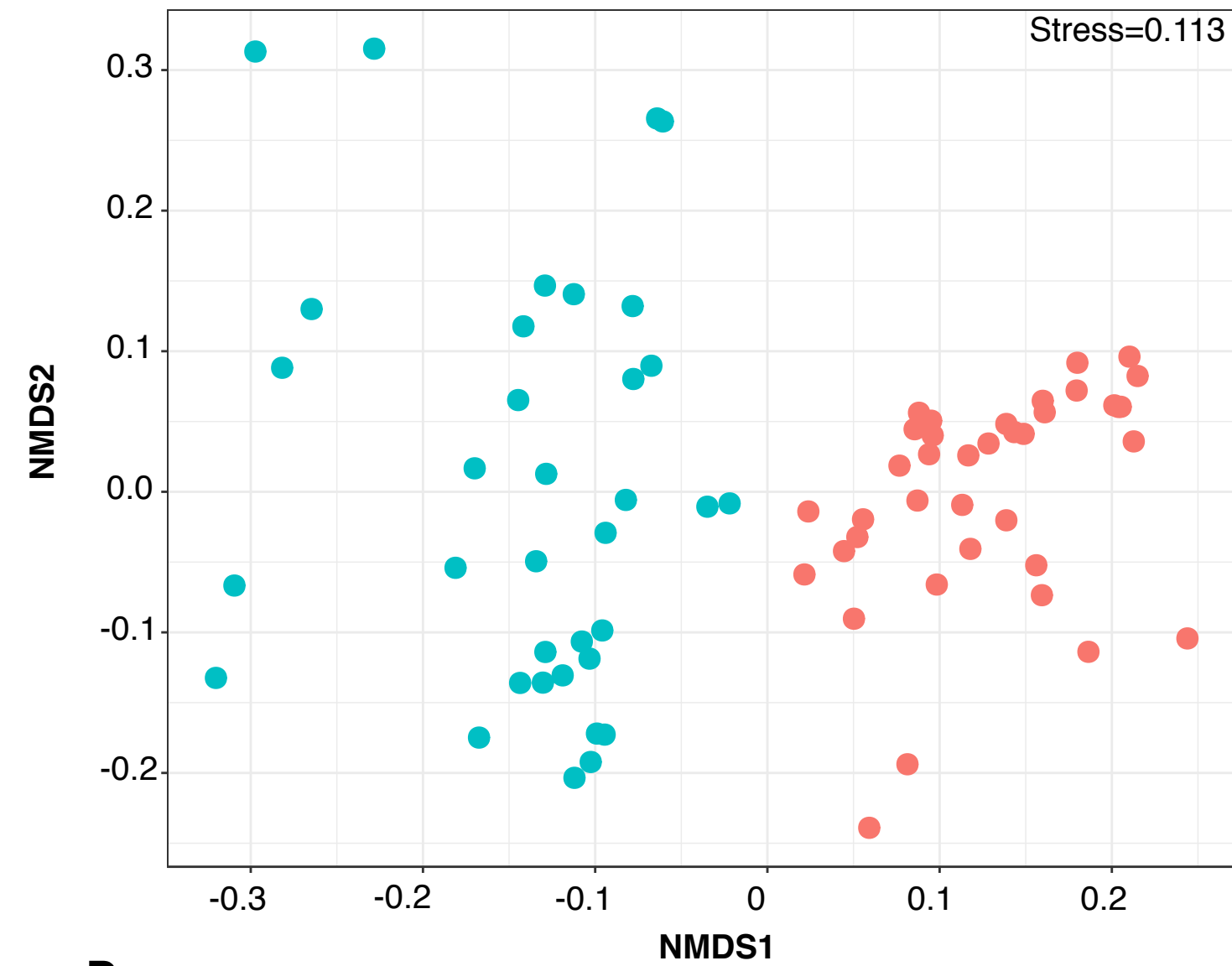
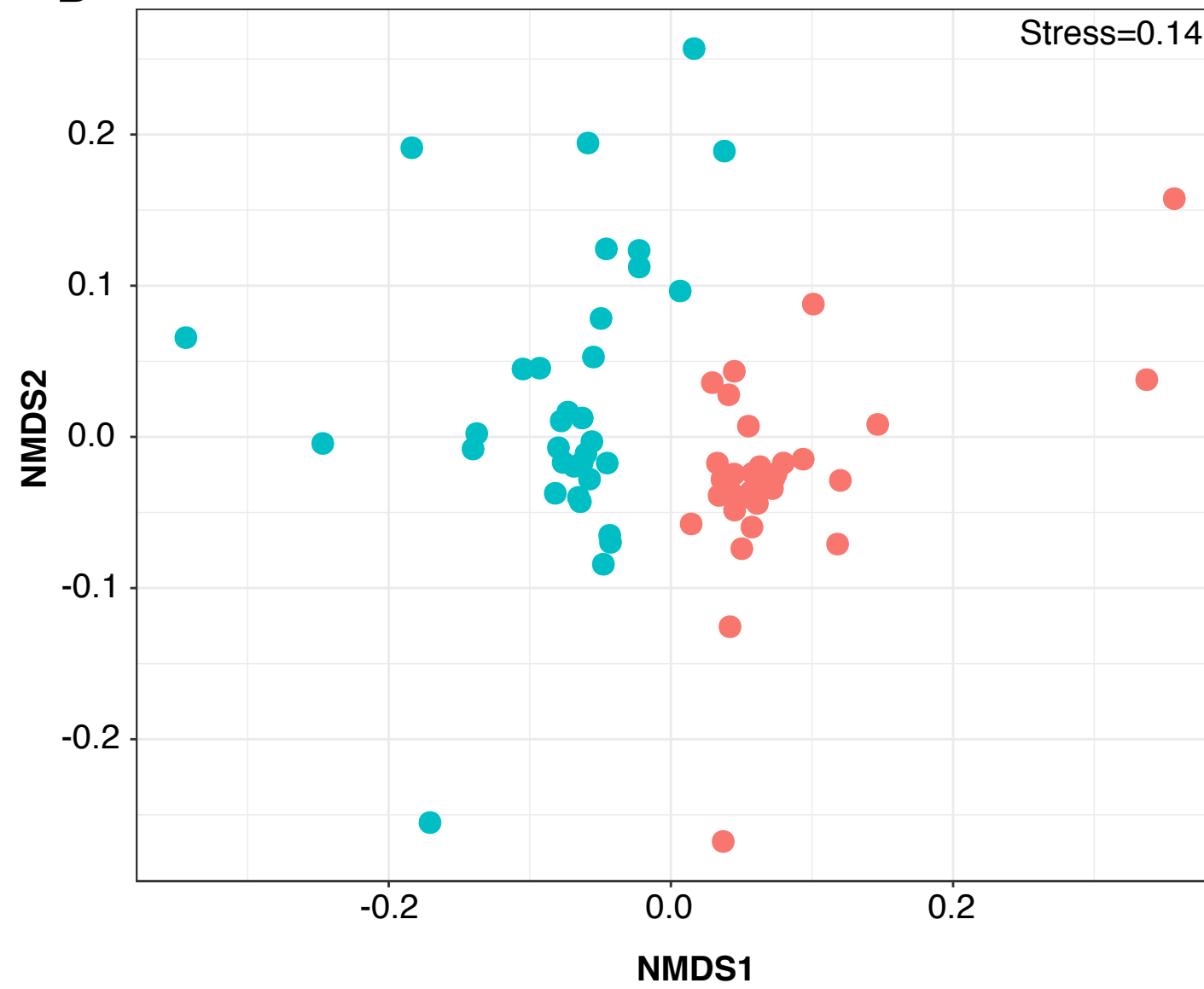
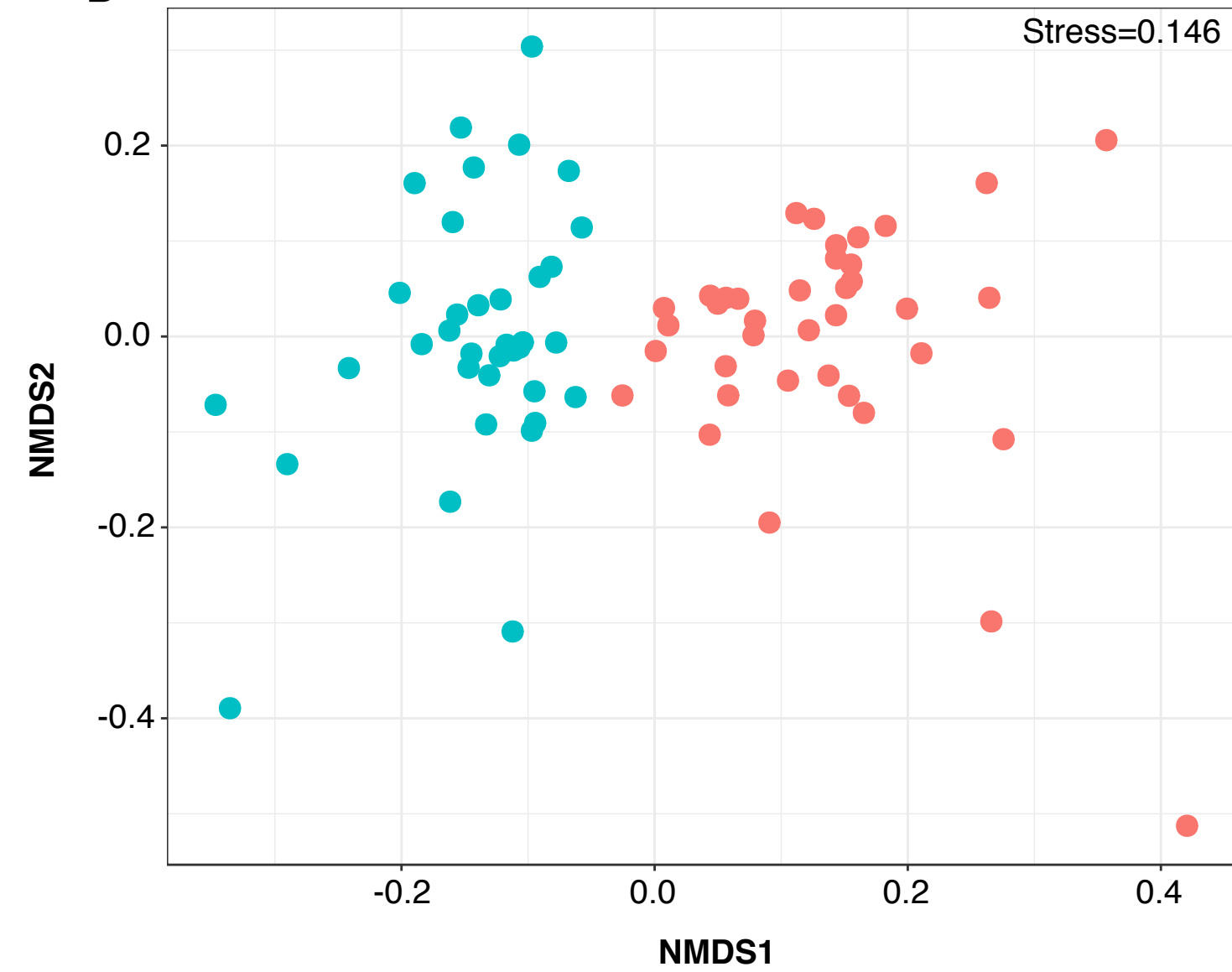
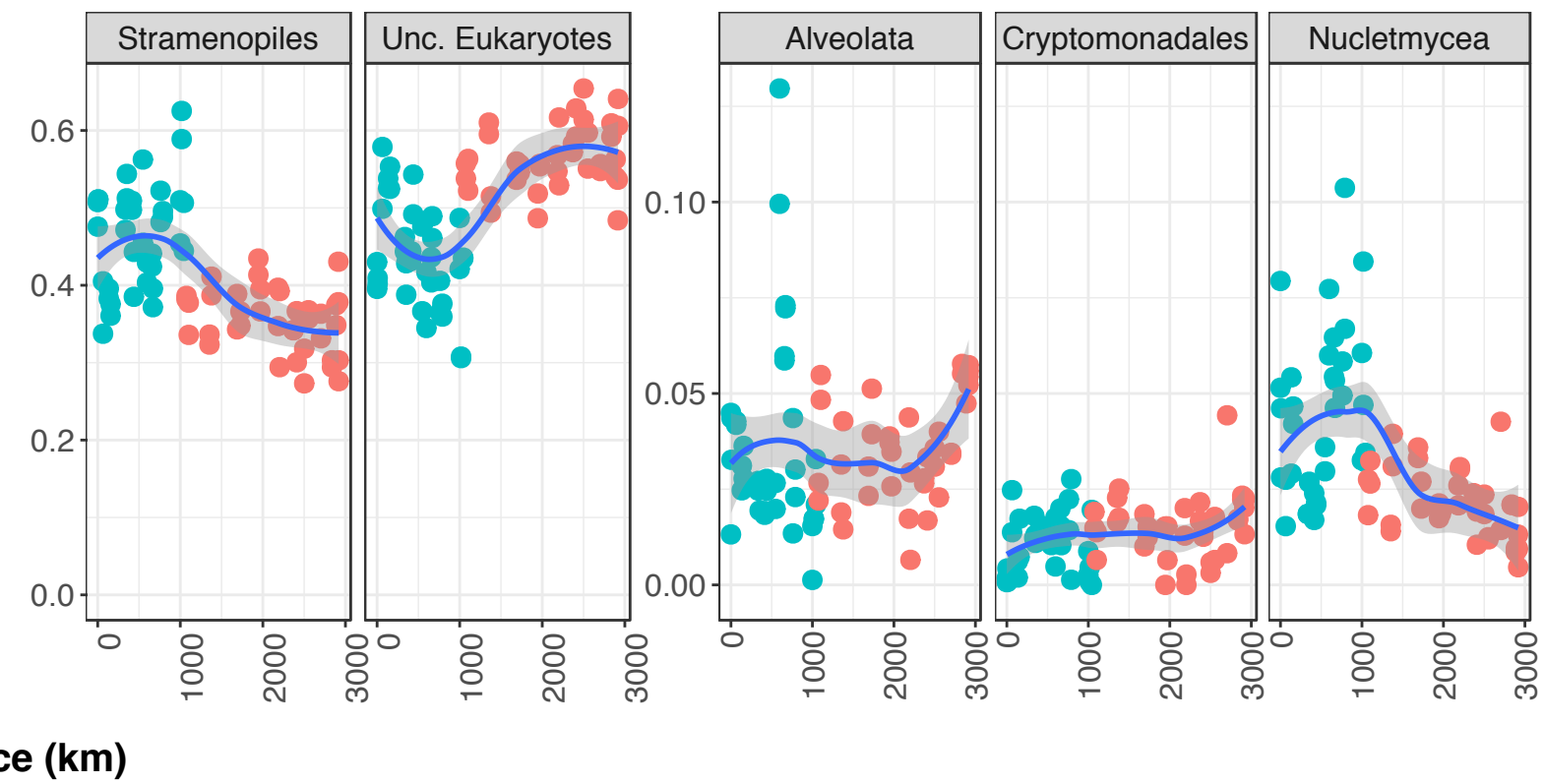
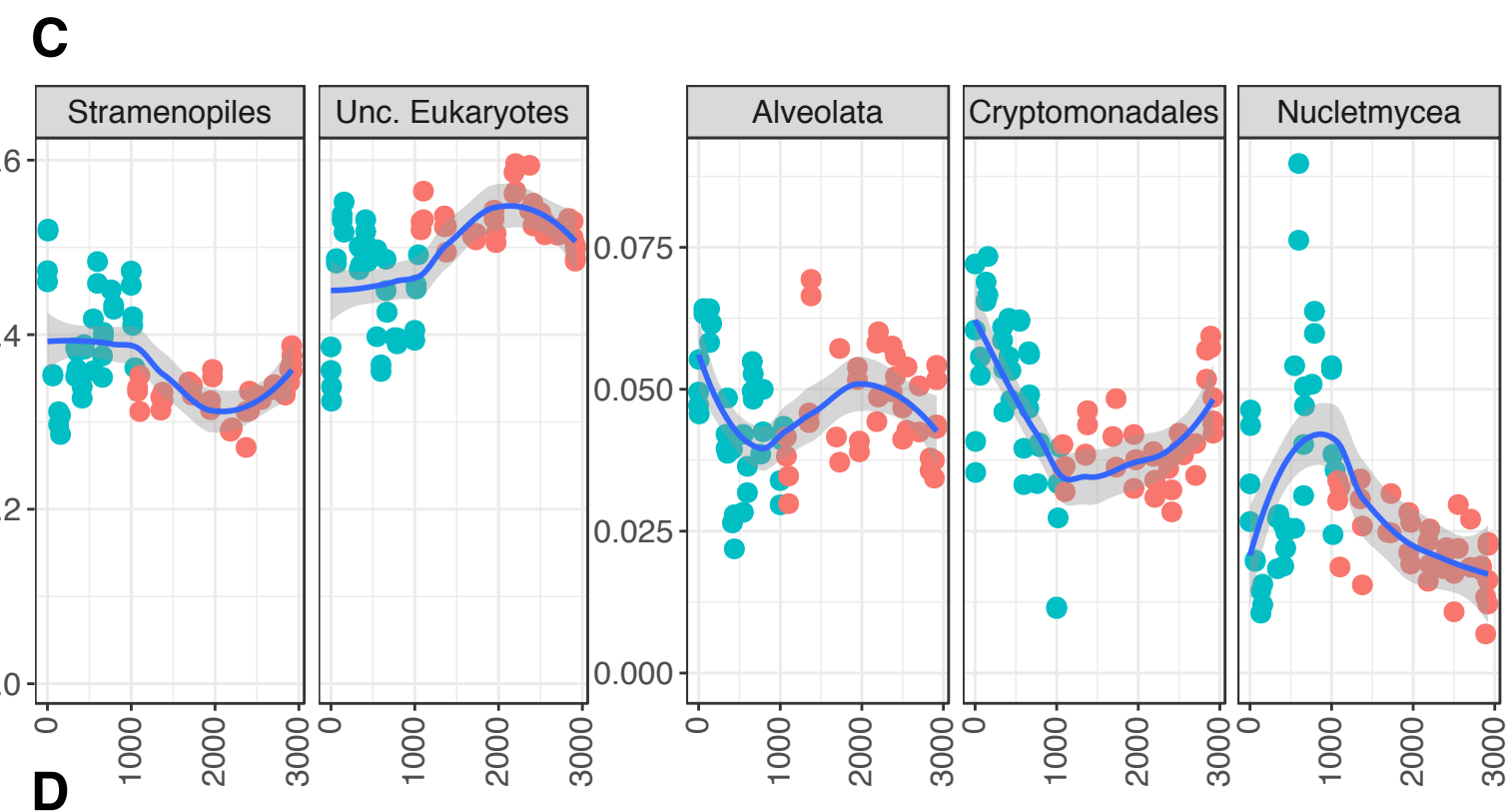
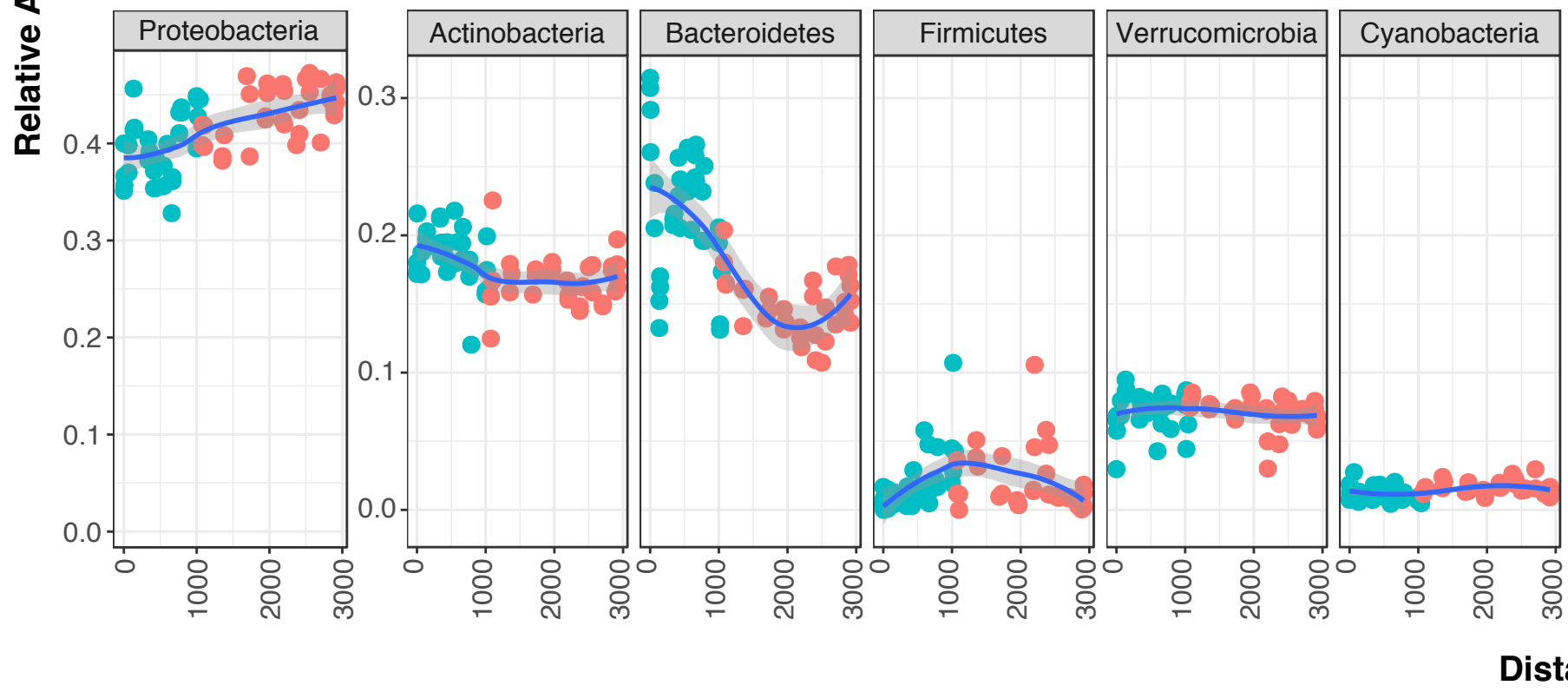
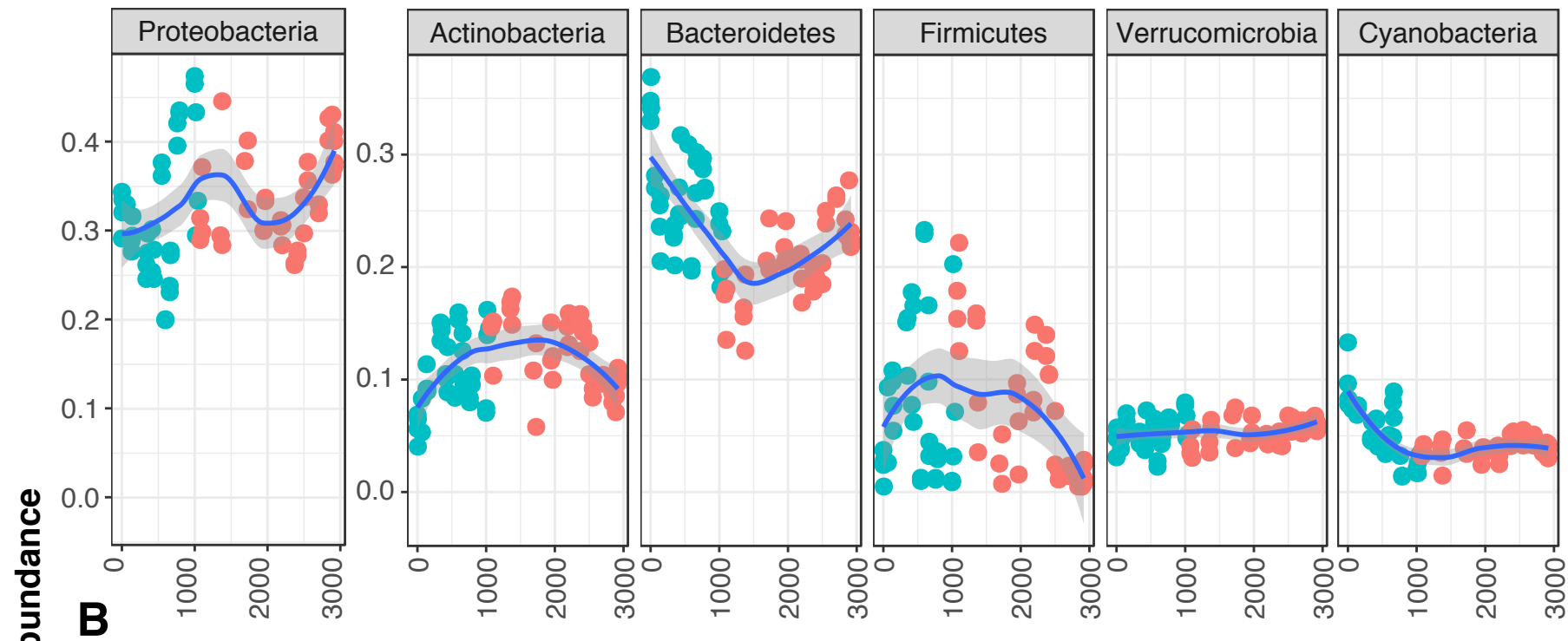
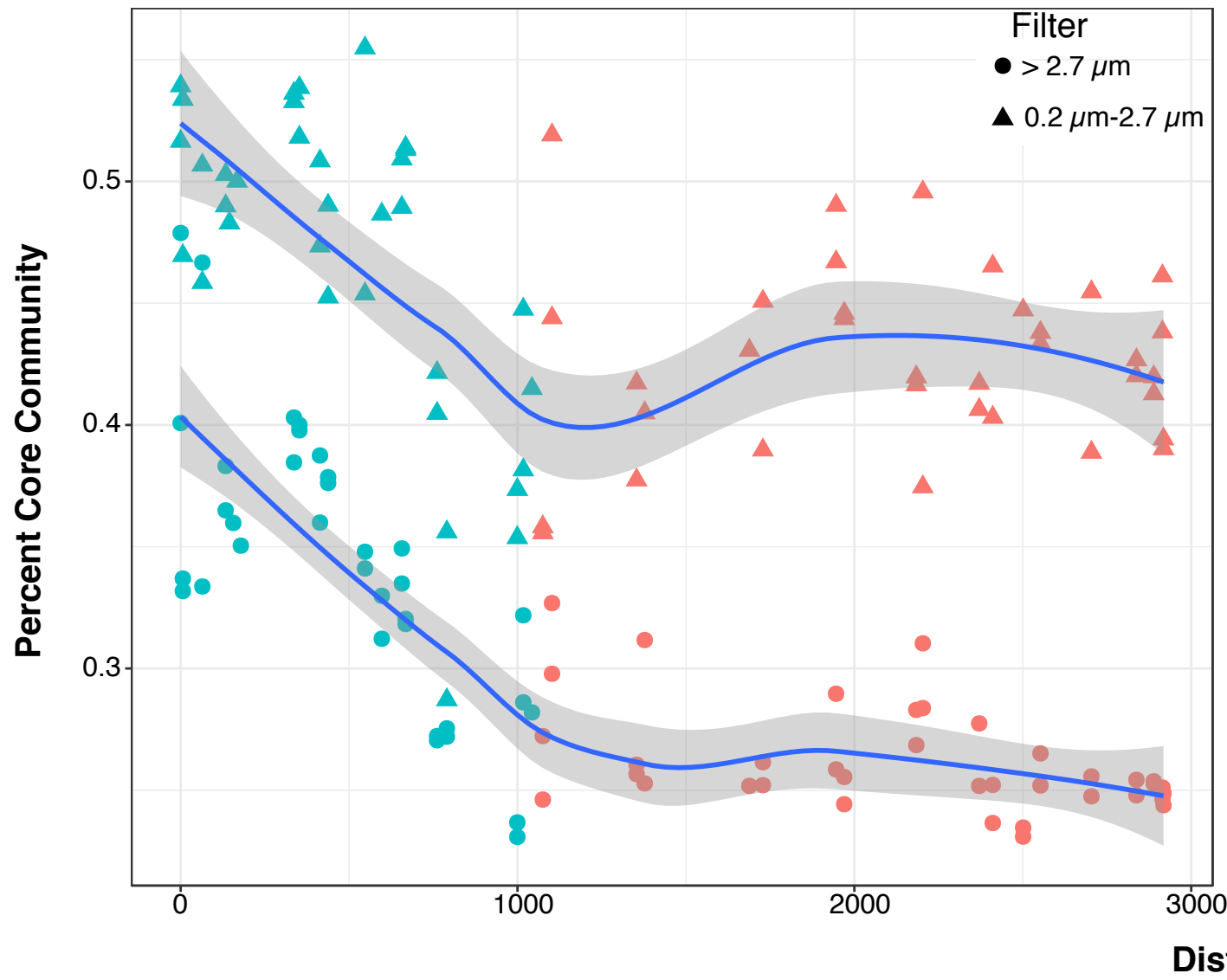
Figure 2A**C****B****D**

Figure 3A



Distance (km)

Figure 4A



B

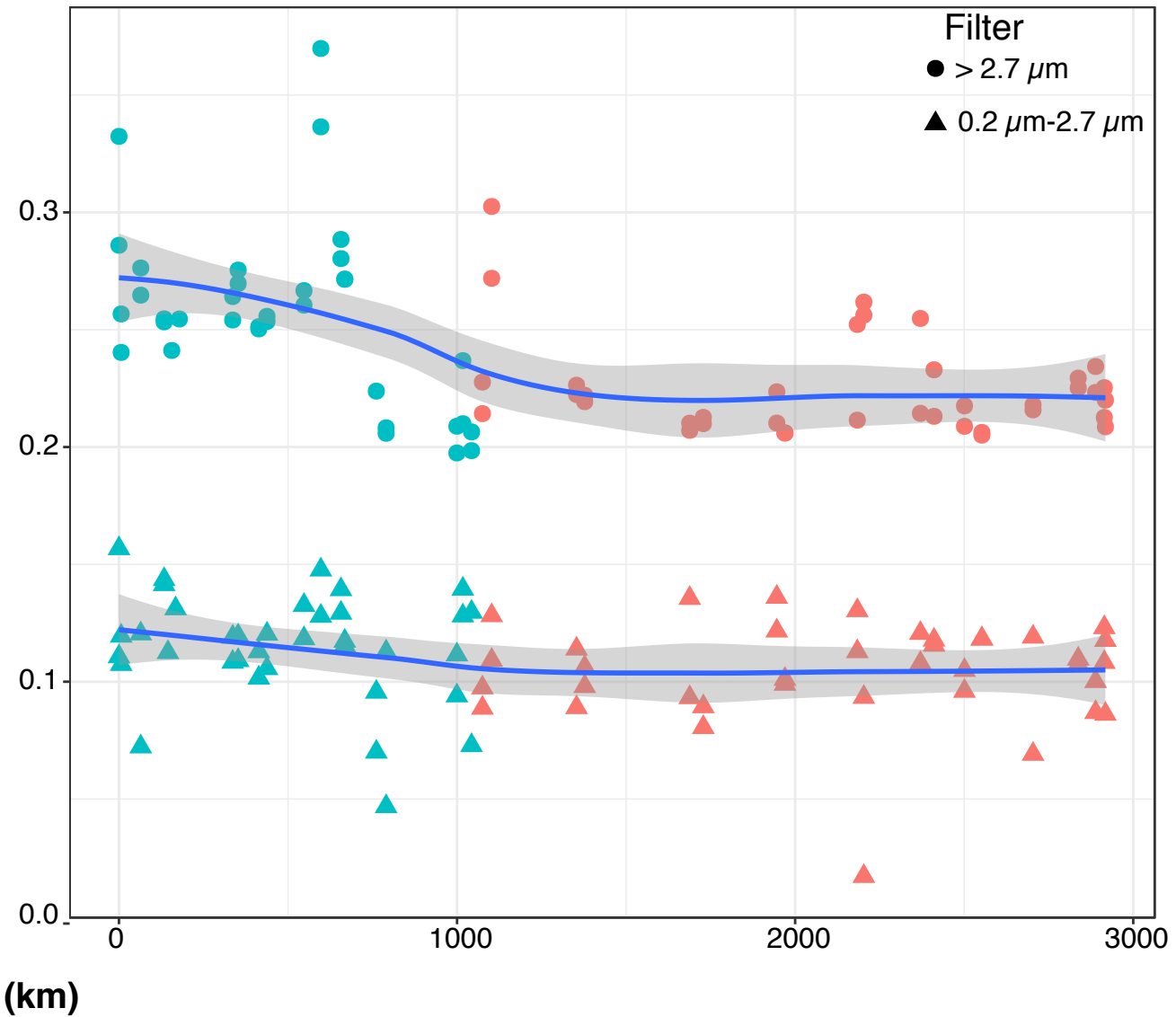


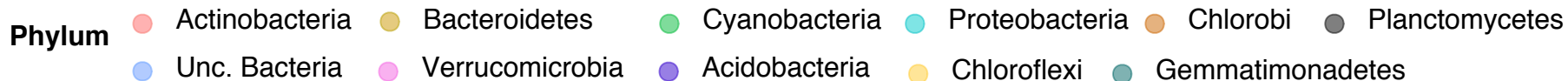
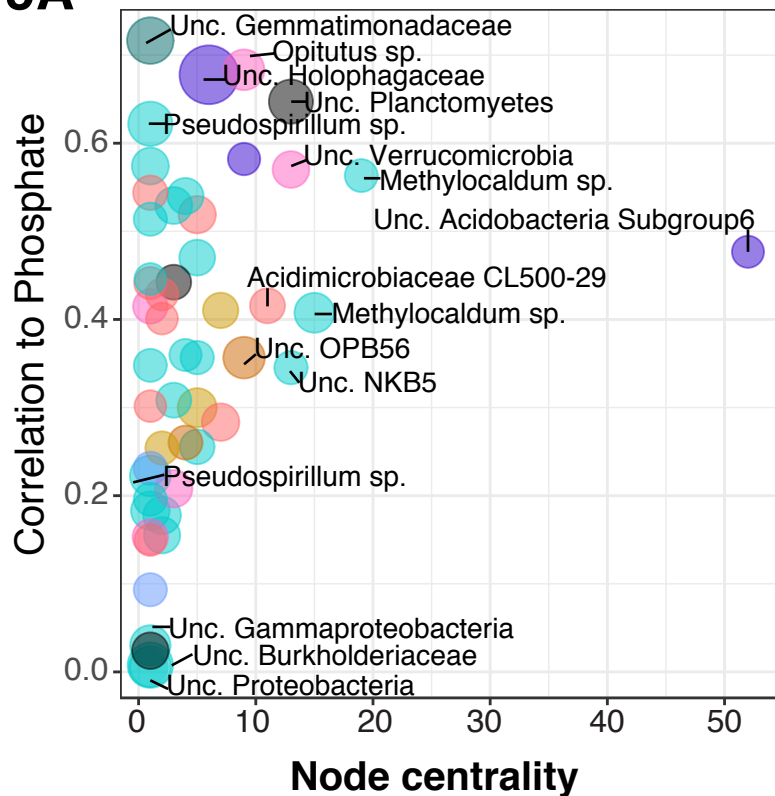
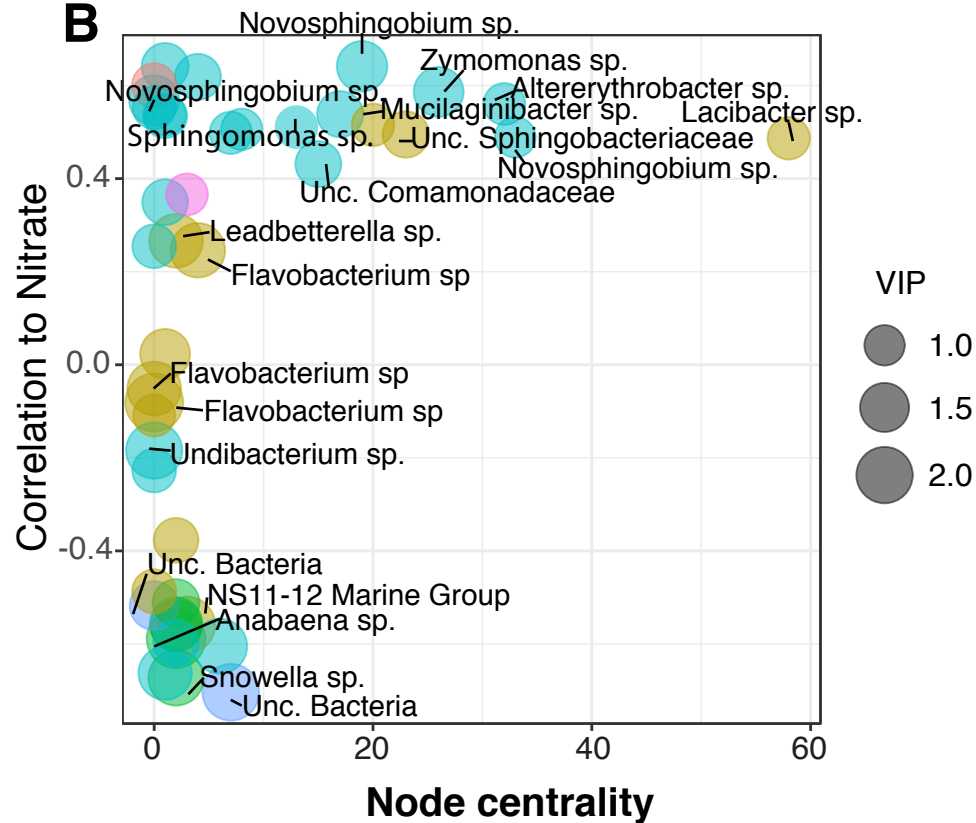
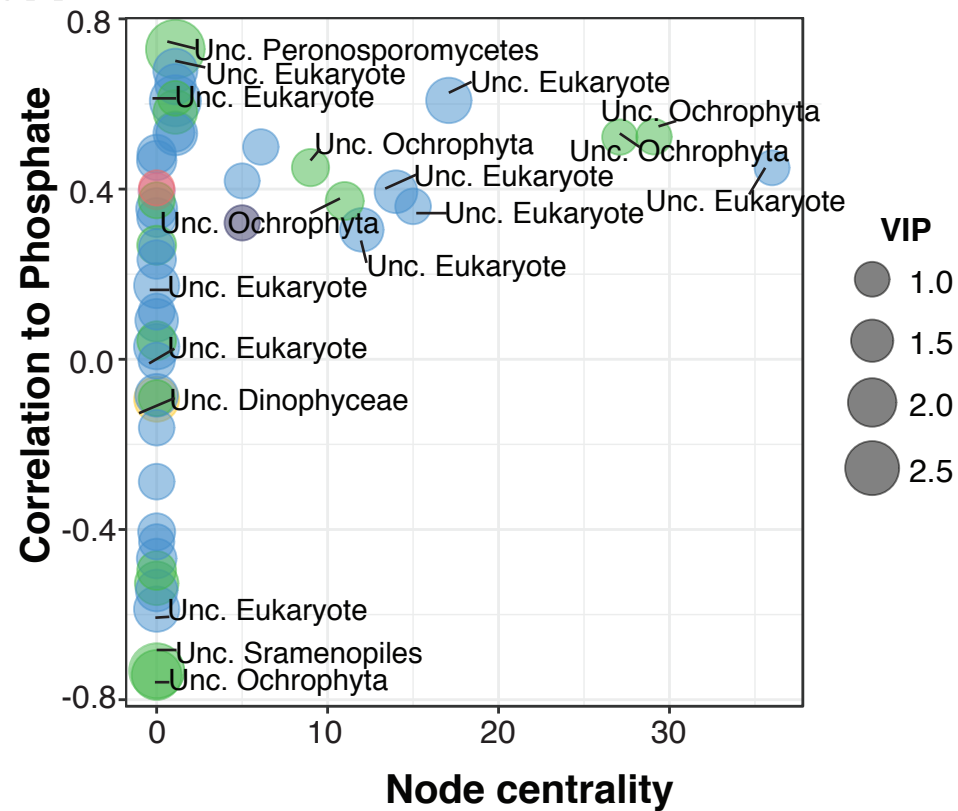
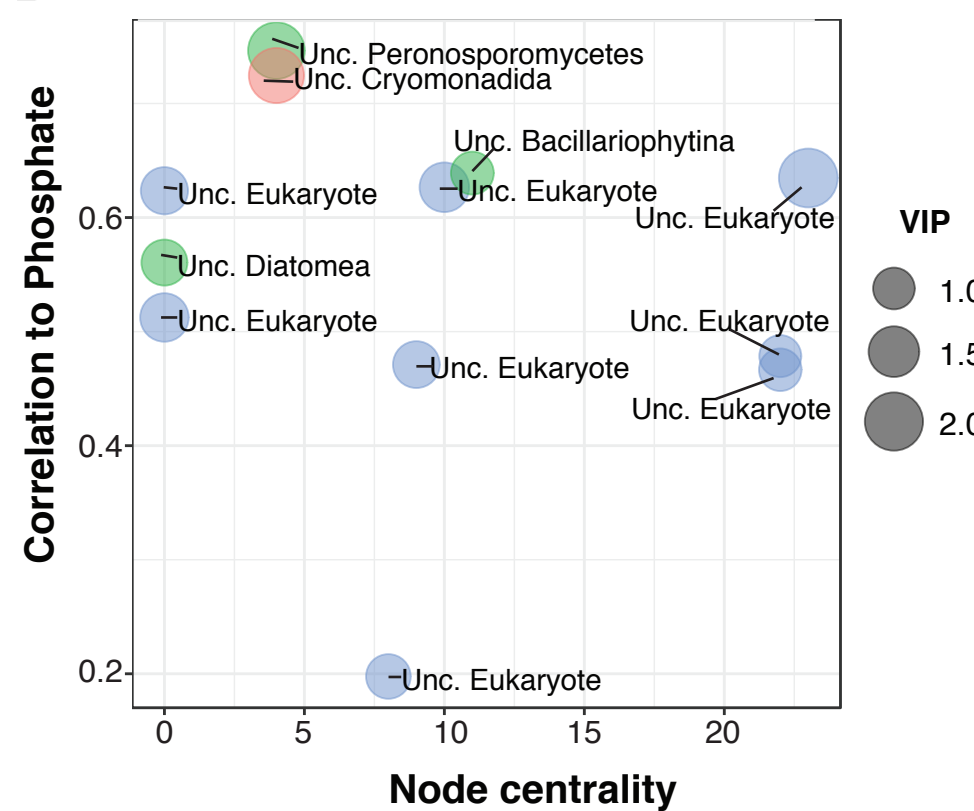
Figure 5A**B**

Figure 6A**B****C**