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1 # Microbial community structure and microbial networks correspond
2 # to nutrient gradients within coastal wetlands of the Great Lakes.
3
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Abstract

Microbial communities within the soil of Great Lakes coastal wetlands drive biogeochemical cycles and provide several other ecosystems services. However, there exists a lack of understanding of how microbial communities respond to nutrient gradients and human activity in these systems. This research sought to address the lack of understanding through exploration of relationships between nutrient gradients, microbial community diversity, and microbial networks among coastal wetlands throughout the Great Lakes. Significant differences in microbial community structure were found among coastal wetlands within the western basin of Lake Erie and all other wetlands studied (three regions within Saginaw Bay and one region in the Beaver Archipelago). Further, within Lake Erie wetlands, chemical and biological structure did not vary with increasing soil depth. Beyond this, alpha diversity levels were highest within Lake Erie coastal wetlands. These diversity differences coincided with higher nutrient levels within the Lake Erie region. Site-to-site variability also existed within the majority of the regions studied, suggesting site-scale heterogeneity may impact microbial community structure. Several subnetworks of microbial communities and individual community members were related to chemical gradients among wetland regions, revealing several candidate indicator communities and taxa which may be useful for Great Lakes coastal wetland management. This research provides an initial characterization of microbial communities among Great Lakes coastal wetlands and demonstrates that microbial communities could be negatively impacted by anthropogenic activities. Anthropogenic impacts to these coastal wetland communities could influence natural biogeochemical cycles which occur within coastal wetland soils, and by extension would directly influence the Great Lakes themselves.

1. Introduction

The Laurentian Great Lakes of North America are one of the largest freshwater systems on Earth, and are critical in supporting biogeochemical cycles, freshwater resources, biodiversity, and economic viability of the surrounding region. Notably, the Great Lakes region has been impacted by anthropogenic pressure, with cumulative stress having a particular impact on the western basin of Lake Erie (Danz *et al*., 2007; Uzarski *et al*., 2017). These negative impacts extend to ecological transition zones between upland and aquatic environments in the form of coastal wetlands which border the Great Lakes (Uzarski, 2009), as agricultural runoff, atmospheric deposition, and urbanization influence water chemistry, thereby reducing water quality and impairing wetlands (Trebitz *et al*., 2007; Morrice *et al*., 2008). This has stoked a surge in research assessing biodiversity and anthropogenic pressure on coastal wetlands of the Great Lakes since the Great Lakes Water Quality Agreement (GLWQA) was established in 1972 (Hackett *et al*., 2017). While much research on coastal wetlands has flourished in the wake of this international agreement, microbial communities within Great Lakes coastal wetlands remain almost entirely uncharacterized (Hackett *et al*., 2017). The few research studies on microbial communities in Great Lakes coastal wetlands have focused on the use of microbial enzymatic assays as a tool to explore decomposition rates and nutrient limitation (Jackson *et al*., 1995; Hill *et al*., 2006). Community diversity, structure, and taxonomic composition have been largely overlooked. As the microbial communities within Great Lakes coastal wetlands have yet to be fundamentally described, it is important to gather baseline data on what microbes exist within these systems, to elucidate how

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65 these microbes could be interacting, and to determine to what extent microbial diversity may
66 already be impacted by anthropogenic chemical disturbance.
67
68 Microbial communities contribute substantially to the ecological functioning of coastal
69 wetlands, and these wetlands are vital in the retention of chemical pollutants (e.g., heavy metals),
70 sediments, and excess nutrients (e.g., N and P) caused by anthropogenic activity from impacting
71 the Great Lakes themselves (Wang & Mitsch, 1998; Sierszen *et al.*, 2012). Coastal wetlands border
72 much of the Great Lakes coastline, where they make up nearly 200,000 ha of habitat between the
73 United States and Canada, despite reduction of habitat by approximately 50% since European
74 colonization (Dahl, 1990; Hecnar, 2004; Sierszen *et al.*, 2012). Further, the economy of the Great
75 Lakes is contingent on the existence and proper functioning of coastal wetlands. In providing
76 ecosystem services and promoting biodiversity, these wetlands have an estimated annual worth of
77 $69 billion USD; the value of recreational fishing alone is valued at $7.4 billion USD per year
78 (Krantzberg & de Boer, 2008; Campbell *et al.*, 2015). As such, negative anthropogenic impacts on
79 microbial communities could influence the economic viability of the Great Lakes region,
80 biodiversity retention, and the functioning of critical elemental cycles which commonly occur
81 within freshwater wetlands.
82
83 Most notably, carbon mineralization occurs within wetland soils via redox processes
84 mediated by microbial communities, and these processes contribute to pollution mitigation and
85 atmospheric greenhouse gas flux (Conrad, 1996; Reddy & DeLaune, 2008). Wetland soils often
86 become chemically structured with increasing depth through sequential reduction of electron
87 acceptors that decrease in metabolic favorability to microbes due to thermodynamic constraints
88 (Conrad, 1996; Reddy & DeLaune, 2008; Kögel-Knabner *et al.*, 2010). As microbial community
89 metabolism changes in concert with soil chemical profiles, microbial community compositional
90 shifts commonly reflect functional changes of the community (Lüdemann *et al.*, 2000; Edlund *et*
91 *al.*, 2008; Lipson *et al.*, 2015). However, while availability of electron acceptors may influence
92 chemical and biological structure within wetland soils, concentration of carbon electron donors
93 can influence the vertical stratification of redox processes (Achtnich *et al.*, 1995; Alewell *et al.*,
94 2008), and by extension, vertical microbial community structure (defined as relative proportions
95 of microbial taxa within a community). As an example of how this may apply to natural
96 environments, increased carbon and nutrient influx from anthropogenic activities (such as
97 agricultural pressure) may impact microbial community structure within coastal wetlands. Impacts
98 to microbial community composition may extend to shifts in chemical cycles and redox processes
99 as consequence, as disturbance to microbial community structure can often lead to a shift in
100 community function (Shade *et al.*, 2012). However, while community structure may be indicative
101 of environmental gradients within wetlands, taxonomic identification of microbes which respond
102 to human pressures is necessary to appreciate which fraction of wetland microbial communities
103 are most sensitive to environmental disturbances.
104
105 Networks of microbial taxa exist within microbial communities, and impacts to individual
106 members could affect entire networks (Faust & Raes, 2012). Thus, it is important explore
107 hypothetical microbial networks within natural environments, and their relationships to changing
108 environmental conditions. Understanding how microbial networks respond to physicochemical
109 shifts could aide in predicting how a future change in environmental conditions (perhaps caused
110 by anthropogenic activity) may impact local microbial communities. Further, identifying microbial

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111 taxonomic and diversity responses to environmental stressors caused by human activity is the first
112 step in developing biological indicators that can predict levels of anthropogenic stress on natural
113 environments, such as wetlands. Physicochemical and biological indicators have been
114 continuously developed to determine which biological taxa are most sensitive to anthropogenic
115 pressures within freshwater wetlands, and by extension, how these biological responses can inform
116 scientists and managers about the health of coastal wetlands along the Great Lakes (Uzarski *et al*.,
117 2017). These indices have been established for physical and chemical attributes (such as nutrient
118 levels, urbanization, land use, etc.), as well as several eukaryotic taxonomic groups (e.g.,
119 macrophytes, macroinvertebrates, fish, anurans, and birds) (Uzarski *et al*., 2017). However, as
120 different taxonomic indicators highlight unique pressures on wetland systems, indicators based on
121 different biological groups can often conflict in their assessment of wetland ecosystem health. As
122 such, it is necessary to examine a wide range of biological indicators to assess different aspects of
123 wetland ecosystem health. A biological index for bacteria and archaea has yet to be developed for
124 responses to human impacts within freshwater coastal wetlands (Uzarski et al., 2017). A first step
125 in establishing a microbial index is to uncover specific networks of microbial taxa (Sims *et al*.,
126 2013; Urakawa & Bernhard, 2017) and diversity patterns found to be related to environmental
127 gradients linked to anthropogenic activity (e.g., soil nutrient levels) among Great Lakes coastal
128 wetlands.
129
130 This study sought to provide an initial characterization of microbial communities within
131 soils of Great Lakes coastal wetlands bordering the western basin of Lake Erie, Saginaw Bay of
132 Lake Huron, and northern Lake Michigan. Wetland sites explored in this study have been
133 extensively researched over multiple years and vary widely in the degree to which they are
134 impacted by human activity (Uzarski *et al*., 2017). This study explored how environmental
135 gradients among these coastal wetlands were related to microbial community structure among
136 wetlands. Additionally, relationships among microbial communities and changing environmental
137 conditions with increasing soil depth were also explored within each wetland site. It was predicted
138 that microbial community structure would be related to environmental gradients among and within
139 coastal wetland regions of the Great Lakes, and elevated nutrient levels within wetlands would
140 decouple the relationship between microbial community structure and soil depth with respect to
141 coastal wetlands lower in nutrient levels, as has been suggested in previous studies (Achtnich *et*
142 *al*., 1995; Alewell *et al*., 2008). Through high-throughput sequencing of the 16S rRNA gene and
143 microbial network analyses, variations in key microbial taxa and subcommunities related to
144 environmental gradients established by wetlands were identified.
145
146 **2. Material and Methods**
147
148 In the summer of 2014, wetland soil cores were collected within Laurentian Great Lakes
149 coastal wetland ecosystems across several sites within several regions. Specifically, soil cores were
150 collected from ten sites across five regions, including two sites in the western basin of Lake Erie
151 (LE), three sites in eastern Saginaw Bay (ESBT), two sites in northern Saginaw Bay (NSB), two
152 sites in western Saginaw Bay (WSB) in Lake Huron, and one site in the Beaver Island archipelago
153 (BA) in Lake Michigan (Fig. 1). These sites were selected as they corresponded to environmental
154 gradients, as well as human impact gradients based upon SumRank scores (an index assessing land
155 use and water quality) as described in Uzarski *et al*. (2017) (Supplemental Fig. 1). Soil cores were
156 collected by hand-driving plastic core tubes (~ 5 cm diameter) vertically into the soil. Among

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157 wetlands, samples were collected within the same vegetation zone across sites (either dominated
158 by cattails, genus *Typhus*, or bulrush, genus *Shoenoplectus*) as an attempt to control for collection
159 bias, as different vegetation zones can harbor microbial communities distinct from other vegetation
160 zones (Tang *et al*., 2011). Cores were sampled to a depth of at least 6 cm (except for one core
161 which was sampled to a depth of 4 cm) and were immediately flash frozen in a dry ice ethanol
162 bath. Samples were transported on dry ice to Central Michigan University wherein they were
163 stored at -80 ˚C.
164
165 Triplicate cores were taken at five wetland sites while duplicate cores were taken at five
166 other wetland sites. For sample extraction and sectioning, cores were extruded while still frozen
167 via a custom-built core extruder. The edge of the core was warmed with a heat gun to allow the
168 soil core to pass efficiently through the plastic container, however, the inner-core did not thaw
169 during extrusion. Ice was applied to the plastic core liner to prevent accelerated thawing. Beginning
170 from the top surface of soil, 2 cm sections were cut via an ethanol and flame-sterilized hacksaw
171 blade and the sectioned core samples were placed into Whirl-Pak bags and stored at -80 ˚C. The
172 extruder face plate was sterilized between cuts of the same core with ethanol. The extruder device
173 was fully cleaned and sterilized between cores with physical scrubbing and ethanol sterilization.
174

175 **2.1 Microbial community analysis**

176
177 Each soil sample was analyzed independently for microbial community analyses. DNA
178 was extracted from ~ 0.25 g of soil using a MoBio PowerSoil DNA Isolation Kit (Mo Bio,
179 Carlsbad, CA) following the standard manufacturer's protocol. Concentrations of extracted DNA
180 were assessed using a Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA) to ensure
181 successful DNA extraction and quantification for sequence library preparation. DNA samples were
182 sent to Michigan State University (East Lansing, MI) for library preparation and sequence analysis
183 at the Research Technology Support Facility. The V4 region of the 16S rRNA gene was amplified
184 for downstream sequencing with the commonly used primers 16Sf-V4 (515f) and 16Sr-V4 (806r)
185 and a previously developed protocol (Caporaso *et al*., 2012; Kozich *et al*., 2013). Paired-end 250
186 bp sequencing was accomplished via a MiSeq high-throughput sequencer (Illumina, San Diego,
187 CA). Acquired DNA sequences were filtered for quality and analyzed using MOTHUR v 1.35.1
188 (Schloss *et al*., 2009) following the MiSeq SOP (available at https://www.mothur.org/) with
189 modifications. Scripts used for sequence processing can be found at the GitHub repository
190 associated with this study (https://github.com/horto2dj/GLCW/). Briefly, paired end sequences
191 were combined into single contigs. Sequences that contained homopolymers > 8 bases, and those
192 less than 251 or greater than 254 bp were removed. Sequences were aligned against the Silva (v
193 119) rRNA gene reference database (Quast *et al*., 2012). Sequences which did not align with the
194 V4 region were also subsequently removed from analysis. Chimeric DNA was searched for and
195 removed via UCHIME (Edgar *et al*., 2011). Sequences were classified via the Ribosomal Database
196 Project (training set v 9; Cole *et al*., 2013) with a confidence threshold of 80. Sequences classified
197 as chloroplast, mitochondria, eukaryotic, or unknown were removed. Remaining sequences were
198 clustered into Operational Taxonomic Units (OTUs) at 0.03 sequence dissimilarity using the
199 opticlust clustering algorithm. Sequence data associated with this research have been submitted to
200 the GenBank database under accession numbers SRR6261304 – SRR6261377 (Horton et al.,
201 2017).
202

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203 **2.2 Chemical analysis**

205 Each soil layer (top, middle, and bottom) was analyzed separately for local chemistry at 206 each site. Within each site, soil samples of the same depth (i.e., top, middle, and bottom soil 207 samples) among duplicate/triplicate cores were combined and homogenized to obtain enough soil 208 for chemical analyses. For chemical analysis, soil samples were sent to Michigan State University 209 Soil & Plant Nutrient Lab (East Lansing, MI) to analyze for percent total N ("TN"), total P ("TP", 210 ppm), total S ("TS", ppm), NO_3^- (ppm), NH_4^+ (ppm), percent organic matter ("OM"), percent 211 organic carbon ("OC"), and C:N. In the field, a YSI multiprobe (YSI Inc., Yellow Springs, OH) 212 was used to measure pH of the water residing directly above each collected soil core.

214 **2.3 Statistical analyses**

216 Statistical analyses were completed using R statistical software version 3.2.2 (R Core 217 Team, 2015) unless otherwise stated. Code used for statistical analyses (and bioinformatic 218 workflow) in this study can be found in the associated GitHub repository 219 (https://github.com/horto2dj/GLCW/).

221 **2.3.1 Physicochemical analysis**

223 Differences in chemical profiles between samples within and among wetland regions were 224 visualized using Principal Component Analysis (PCA). Prior to PCA, percentages were arcsin 225 square root transformed and ratios were log transformed. Additionally, Pearson correlation 226 analyses were performed to search for significant correlations between chemical variables. 227 Collinearity in the dataset was addressed by combining highly correlated environmental variables 228 ($r > 0.7$, $p \leq 0.001$). Only one of the correlated variables was included in PCA to remove 229 exaggeration of correlated variables in PCA structure. Permutational Multivariate Analysis of 230 Variance (perMANOVA; Anderson, 2001) was used to determine the influences of region and soil 231 depth on physicochemical composition of samples, and 95% confidence intervals were established 232 to compare differences among groups. Chemical depth profiles were also visualized for each 233 wetland site to understand shifts in measured environmental variables with increasing soil depth.

235 **2.3.2 Alpha diversity analysis**

237 Alpha diversity analyses were performed to explore variation in OTU richness and 238 evenness among wetland sites, regions, and soil depths, as well as to determine whether observed 239 trends were driven by environmental variables. Prior to alpha diversity analyses, sequence 240 abundance for each sample was subsampled to the lowest sequence abundance for any one sample 241 ($n = 48{,}226$ sequences). Singletons were maintained within the sequence dataset for alpha diversity 242 analyses, as alpha diversity indices can be reliant on the presence of singletons for proper 243 estimation. Alpha diversity was calculated for each site using MOTHUR, including Chao1 244 richness and non-parametric Shannon diversity. Linear mixed-effect models and ANOVAs were 245 used to test influences of wetland site, region, and soil depth on alpha diversity, controlling for 246 wetland site as a random effect. Linear models and ANOVAs were used to test for variation in 247 alpha diversity among wetland sites. If significant variation was found within an ANOVA result, 248 post-hoc comparisons were implemented between sample groups using Tukey's Honest

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249 Significant Differences (HSD) tests with Bonferroni adjustments (p-values obtained by number of
250 comparisons) for pairwise comparisons.
251
252 **2.3.3 Beta diversity analysis**
253
254 Beta diversity analyses were used to evaluate variation in microbial community structure
255 among wetland sites, regions, and soil depths, and to assess the extent to which observed variation
256 was explained by environmental conditions. Singletons and doubletons were removed from the
257 dataset for beta diversity analyses. All sequence data were maintained for beta diversity analyses
258 and transformed using the *DeSeq2* (Love *et al*., 2014) package, which normalized OTU
259 abundances among samples using a variance stabilizing transformation (VST) (McMurdie &
260 Holmes, 2014). The *phyloseq* (McMurdie & Holmes, 2013) and *Vegan* (Oksanen *et al*., 2007)
261 packages were used to compare beta diversity among samples. Dissimilarity in microbial
262 community structure among samples within and among sites was visualized using Non-metric
263 Multidimensional Scaling (NMDS) plots based on pairwise Bray-Curtis dissimilarity estimates.
264 The function *envfit* of the Vegan package was used to evaluate correlation between chemical
265 parameters and microbial community structure among samples according to NMDS. "Depth" was
266 also implemented as a dummy variable to test correlation between depth and microbial community
267 structure.
268
269 To test for significant differences in beta diversity among wetland sites, regions, and soil
270 depth, perMANOVA were implemented. Specifically, these tests evaluate significant variation
271 among within group and between group means (Clarke, 1993; Anderson, 2001; Anderson &
272 Walsh, 2013). If perMANOVA found significant differences among groups at the global level,
273 pairwise perMANOVA tests between groups were implemented with Bonferroni significance
274 adjustments to control for multiple pairwise comparisons. Anderson's permutation of dispersions
275 test (PERMDISP; Anderson, 2006; Anderson *et al*., 2006) was used to test for differences in
276 variance of community structure among sample groups (i.e. sites, regions, soil depths). Tukey's
277 Honest Significant Difference (HSD) tests were implemented with adjusted p-values for multiple
278 pairwise comparisons if significant differences in dispersion were found among groups.
279
280 To explore relationships between regional microbial community structure and
281 environmental variables, NMDS plots were generated for each individual region. Applying NMDS
282 to each region also allowed for the assessment of the correlational relationship between community
283 structure and soil depth (as a dummy variable) and other environmental variables (using the *envfit*
284 function) within individual regions. To test for differences in microbial community structure
285 between/among sites within a region, as well as among depths within a region, perMANOVA was
286 implemented individually for each region.
287
288 **2.3.4 Network analyses**
289
290 To explore relationships between microbial sub-communities and individual OTUs to
291 environmental variables, Weighted Correlation Network Analysis (WGCNA) was implemented
292 on OTU relative abundances using the *WGCNA* package (Langfelder & Horvath, 2008; Langfelder
293 & Horvath, 2012), executed as previously described (Guidi *et al*., 2016; Henson *et al*., 2016) with
294 modifications. OTUs which did not possess at least 2 sequences across 10% of samples were

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295 removed from network analyses. These OTUs were removed to eliminate OTUs with potentially
296 spurious correlations to environmental variables or other OTUs, as well as to reduce computational
297 stress of analyses. Remaining OTU abundances across samples were normalized using variance
298 stabilizing transformation (VST) performed as described previously for beta diversity analyses.
299 To ensure scale-free topology of the network, the dissimilarity matrix generated through VST was
300 transformed to an adjacency matrix by raising this dissimilarity matrix to a soft threshold power.
301 A threshold power of $p = 4$ was chosen to meet scale-free topology assumptions based upon
302 criterion established by Zhang & Horvath (2005). Scale-free topology of network relationships
303 was further ensured through regression of the frequency distribution of node connectivity against
304 node connectivity; a network is scale-free if an approximate linear fit of this regression is evident
305 (see Zhang & Horvath, [2005] for more in-depth explanation). A topological overlap matrix
306 (TOM) was generated using the adjacency matrix, and subnetworks of highly connected and
307 correlated OTUs were delineated with the TOM and hierarchical clustering. Representative
308 eigenvalues of each subnetwork (i.e., the first principal component) were correlated (Pearson) with
309 values of measured environmental variables to identify the subnetworks most related to said
310 environmental variables. The subnetworks with the highest positive correlations to environmental
311 variables of interest (e.g., NO_3^-, C:N, etc.) were selected for further analyses of relationships among
312 subnetwork structure, individual OTUs, and environmental variables. Partial Least Square
313 regression (PLS) was used to test predictive ability of subnetworks in estimating variability of
314 environmental parameters, which allowed for delineation of potential indicator subnetworks and
315 OTUs. Pearson correlations were calculated between response variables and leave-one-out cross-
316 validation (LOOCV) predicted values. If PLS found that regression between actual and predicted
317 values was below minimum threshold of $R^2 = 0.3$, WGCNA analysis was halted for that network,
318 as the network was deemed to lack predictive ability of that environmental variable. Variable
319 Importance in Projection (VIP) (Chong & Jun, 2005) analysis was used to determine the influence
320 of individual OTUs in PLS. A high VIP value for an OTU indicates high importance in prediction
321 of the environmental response variable for that OTU. For network construction and visualization
322 purposes, the minimum correlation value required between two OTUs to constitute an "edge"
323 between them was delineated at different r values for each network related to an individual
324 environmental variable (ranging between 0.1 – 0.25), as co-correlations between OTUs within
325 some networks were stronger than others. The number of co-correlations an OTU has with other
326 OTUs within a network defines its "node centrality" (as described by Henson *et al.*, 2016).
327
328 **3. Results**
329
330 **3.1 Chemical analyses**
331
332 Significant correlations ($r > 0.7$, $p \leq 0.001$) were found among NH_4^+, OM, OC, and TN.
333 Thus, downstream analyses combined these values into one parameter, "NUTR", represented by
334 OM values as this variable was the most strongly correlated with each of the other variables.
335 Environmental data were analyzed with a PCA and PC1 and PC2 explained 56.2% and 20.6% of
336 the variation among samples, respectively (Fig. b). perMANOVA found significant differences in
337 physicochemical profiles based on region ($R^2 = 0.570$, $p \leq 0.001$) and depth ($R^2 = 0.058$, $p \leq 0.01$).
338 Lake Erie coastal wetlands were chemically distinct from other wetland regions (ESBT and NSB;
339 adjusted p = 0.01) according to perMANOVA and pairwise perMANOVA based on Euclidean
340 distance. Ninety-five percent confidence intervals demonstrated no overlap between Lake Erie

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341 coastal wetlands and other coastal wetlands (Fig. 2). This separation was related to increased
342 NUTR, NO_3^-, and S.
343 Increasing depth within cores showed a consistent shift in environmental variables, except
344 in those sites located in the western basin of Lake Erie (Supplemental Fig. 2). Specifically, OM,
345 OC, and TN consistently decreased with increasing depth within each region except Lake Erie.
346 Similarly, C:N increased with depth in each region except Lake Erie, wherein the C:N ratio
347 remained relatively low (~ 12) and stable with increasing soil depth. Within the Lake Erie wetland
348 region, pH was more acidic in the overlying water with respect to all other wetland regions
349 (Supplemental Table 1). However, pH was still relatively neutral within Lake Erie (average pH =
350 7.26 ± 0.24), whereas other wetland regions (regions within Saginaw Bay and Beaver Archipelago)
351 experienced slightly more basic pH, with average pH among these regions ranging between 7.72
352 – 8.39.
353
354 **3.2 Alpha diversity**
355
356 Sufficient depth of sampling was reinforced by rarefaction curve analysis (Supplemental
357 Fig. 3). Good's coverage values ranged between 89.3 – 93.5% for each region at the subsampled
358 value of 48,226 sequences. Chao1 richness estimates varied significantly among wetland regions
359 ($F = 8.38$, $p \leq 0.05$), as well as wetland sites ($F = 16.78$, $p \leq 0.001$). Pairwise comparisons found
360 that the LE region had significantly higher ($p \leq 0.01$) Chao1 estimates than NSB and WSB regions
361 (Fig. 3; Supplemental Table 2). Additionally, pairwise comparisons found a high degree of
362 significant variability ($p \leq 0.01$) in Chao1 estimates among wetland sites (Supplemental Table 2).
363 Further, Shannon diversity levels also significantly varied among wetland sites ($F = 4.57$, $p \leq$
364 0.001), with site LE_D having significantly higher ($p \leq 0.01$) Shannon diversity levels than sites
365 ESBT_A and WSB_B (Supplemental Table 2). Soil depth did not influence alpha diversity levels.
366
367 Shannon diversity and Chao1 were both positively correlated with measured environmental
368 variables (Table 1). Specifically, Chao1 estimates increased with NO_3^-, P, and S concentrations (p
369 ≤ 0.01), and were weakly positively correlated ($p \leq 0.05$) with NUTR. Additionally, Shannon
370 diversity levels increased alongside NUTR and S ($p \leq 0.001$), and were weakly positively
371 correlated with NO_3^- ($p \leq 0.05$). There were no significant relationships between alpha diversity
372 and C:N, and alpha diversity was not negatively correlated with any of the measured environmental
373 variables.
374
375 **3.3 Beta diversity**
376 **3.3.1 Beta diversity among regions**
377
378 Multivariate analyses were implemented to explore relationships between microbial
379 communities and environmental gradients among wetland regions. NMDS demonstrated
380 separation of microbial communities based on wetland site, region, and soil depth (Fig. 4).
381 Substantiating this result, perMANOVA confirmed that differences in microbial community
382 structure were significantly related to wetland region ($R^2 = 0.220$, $p \leq 0.001$), site ($R^2 = 0.119$, $p \leq$
383 0.001), and soil depth ($R^2 = 0.070$; $p \leq 0.001$). Post-hoc pairwise perMANOVA found that
384 community structure within the LE region was significantly distinct ($p \leq 0.01$) from all other
385 wetland regions (Table 2). No significant differences in community structure were found between
386 any other wetland regions compared. Additionally, microbial community beta diversity was

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387 distinct ($p \leq 0.003$) between the top soil depth and the middle and bottom soil depths. However,
388 no significant differences in microbial community structure were found between the middle and
389 bottom soil depths (Table 2). Variation in microbial community structure was significantly
390 correlated ($p \leq 0.001$) to depth ($r = 0.41$), NO_3^- ($r = 0.20$), NUTR ($r = 0.60$), and S ($r = 0.41$), and
391 also correlated ($p \leq 0.016$) with C:N ($r = 0.11$) and P ($r = 0.14$) (Supplemental Table 3).
392
393 Beta dispersion tests suggested significant variation in structural variance among regions
394 ($p \leq 0.05$), however, Tukey's HSD test using adjusted p-values for multiple comparisons did not
395 find any significance ($p > 0.05$) between pairwise comparisons of regional groups. There were no
396 differences in community structural dispersion among soil depths.
397

398 **3.3.2 Beta diversity within regions**

399
400 Microbial community associations with environmental variables were also explored within
401 regions to examine variation among wetland sites. Individual NMDS plots of each region
402 identified relationships between microbial community structure and several environmental
403 variables using vector-fitting regression, and strengths of these relationships were dependent upon
404 the wetland region explored (Fig. 5; Supplemental Table 3). Depth was significantly related ($p \leq$
405 0.05) to microbial community structure in all wetland regions except NSB and LE. However,
406 microbial community structure may have been more strongly related to depth in NSB ($r = 0.35$, p
407 $= 0.071$) than LE ($r = 0.19$, $p = 0.40$). NUTR was significantly related ($p \leq 0.01$) to community
408 structure within regions BA ($r = 0.82$), ESBT ($r = 0.51$), and LE ($r = 0.66$). C:N was related ($p \leq$
409 0.01) to community structure within regions of Saginaw Bay (i.e., ESBT [$r = 0.65$], NSB [$r = 0.58$],
410 and WSB [$r = 0.58$]). Beta diversity was not significantly associated with concentrations of NO_3^-
411 in any region.
412
413 To test for significant differences in microbial beta diversity within regions, perMANOVA
414 was used to evaluate differences in microbial community structure among soil depths and sites
415 within wetland regions (Supplemental Table 3). Depth did not significantly explain microbial
416 community structure within the region LE ($p = 0.65$), however, it did explain differences in
417 microbial community structure within the other wetland regions, specifically BA ($R^2 = 0.414$; $p =$
418 0.006), ESBT ($R^2 = 0.154$; $p = 0.001$), NSB ($R^2 = 0.161$; $p = 0.093$), and WSB ($R^2 = 0.259$; $p =$
419 0.014). Significant differences in microbial community structure were found among different
420 wetland sites within regions ESBT ($R^2 = 0.192$; $p = 0.001$), LE ($R^2 = 0.236$; $p = 0.004$), and NSB
421 ($R^2 = 0.140$; $p = 0.003$). As only one site was sampled within the BA region, testing for differences
422 among wetland sites within the BA region could not be accomplished.
423

424 **3.4 Network analyses**

425
426 Weighted Correlation Network Analysis (WGCNA) was used to explore strong
427 relationships between subcommunities and individual OTUs with environmental parameters
428 within Great Lakes coastal wetlands. After removal of OTUs that did not have at least two
429 representative sequences in at least 10% of samples, a total of 7,562 OTUs remained for WGCNA.
430 In determining scale-free topology of the OTU network, a soft power threshold of 4 was reached,
431 and an R^2 of 0.87 was established as linear fit from the regression of the frequency distribution of
432 node connectivity against node connectivity (Supplemental Fig. 4). Of the 33 constructed

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433 subnetworks, the same one (subnetwork "orange") was found to be most strongly correlated to
434 both NUTR (r = 0.94) and NO_3^- (r = 0.55) (Supplemental Fig. 5). A separate subnetwork ("pink")
435 was strongly correlated (r = 0.74) to C:N. All correlations of subnetworks to environmental
436 variables were significant ($p \leq 0.001$). OTU VIP values ≤ 1 were removed due to the large amount
437 of OTUs within subnetworks correlated with C:N for visualization purposes.
438
439 For subnetwork relationships to NUTR (including OM, OC, NH_4^+, and TN), partial least
440 squares analysis (PLS) found that 69 OTUs were 93.8% predictive of variance in NUTR
441 (Supplemental Fig. 6). OTU co-correlation networks were constructed using an OTU co-
442 correlation threshold of 0.25, with strong correlations (r > 0.59) between all OTUs and NUTR
443 (Fig. 6). Of the top 15 OTUs contributing to PLS regression by VIP score, seven were related to
444 *Betaproteobacteria*, five were related to *Anaerolineaceae* (within *Chloroflexi*), and one
445 representative OTU was related to each of *Bellilinea* (*Chloroflexi*), *Desulfobacterales*
446 (*Deltaproteobacteria*), and *Rhizobiales* (*Alphaproteobacteria*).
447
448 For subnetwork relationships to C:N, PLS found that 144 OTUs were 59.0% predictive of
449 variance in C:N (Supplemental Fig. 7). Networks were constructed using an OTU co-correlation
450 threshold of 0.1, within positive or negative correlations (r > +/- 0.2) between OTUs (VIP > 1) and
451 C:N (Fig. 7). Of the top 15 OTUs by VIP score within the network, two OTUs related to
452 *Bacteroidetes* were negatively correlated with C:N. Other top OTUs were positively related to
453 C:N, including seven OTUs related to *Anaerolineaceae*, four OTUs which were unclassified
454 *Bacteria*, and one representative OTU related to each of *Bacillus* (*Firmicutes*) and *Chloroflexi*.
455

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456 **4. Discussion**
457
458 **4.1 Microbial diversity driven by chemistry within Great Lakes coastal wetlands**
459
460 This study is the first to suggest that anthropogenic disturbance patterns correspond to
461 microbial community differences in Great Lakes coastal wetlands as consistent with other
462 taxonomic groups such as plants, birds, fish, and invertebrates (Howe *et al*., 2007; Tulbure *et al*.,
463 2007; Uzarski *et al*., 2009; Cooper *et al*., 2012; Uzarski *et al*., 2017). Microbial community
464 structure was significantly dissimilar between LE and all other wetland regions, and these
465 differences were related to physicochemical differences among coastal wetlands (Fig. 2, Fig. 4,
466 Table 2). As the wetlands within the Lake Erie region maintained the highest nutrient
467 concentrations within the soil, it is possible that anthropogenic stressors related to nutrient loading
468 (and potentially other pollutants) could be driving structural differences in microbial communities
469 among Great Lakes coastal wetlands. Further, network analysis found several taxa/sub
470 communities that were highly correlated to nutrient levels across wetlands explored in this study.
471 Previous research has found that nutrient levels (e.g., C, N, P, etc.), to varying degrees, can
472 influence microbial community composition and structure (Hartman *et al*., 2008; Peralta *et al*.,
473 2013; Ligi *et al*., 2014; Arroyo *et al*., 2015). Lake Erie coastal wetlands (and the watershed which
474 drains into them) have been historically impacted by anthropogenic pollution and agricultural
475 practices, particularly in comparison to other coastal wetlands within the Laurentian Great Lakes
476 region. This has been demonstrated by multiple ecological indices (e.g., Cvetkovic & Chow-
477 Fraser, 2011; Uzarski *et al*., 2017) and physicochemical uniqueness (increased levels of nutrients
478 and particulate matter) within the western basin of Lake Erie (Danz *et al*., 2007; Trebitz *et al*.,

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479 2007; Cvetkovic & Chow-Fraser, 2011; Uzarski *et al*., 2017). Data presented in this study
480 corroborate this historical evidence of human impact and nutrient loading in the western basin of
481 Lake Erie (Fig. 2., Supplemental Fig. 1), which may be influencing the Lake Erie wetlands
482 explored in this study.
483
484 High nutrient influx could also be influencing the chemical and microbial vertical structure
485 within coastal wetland soils. Microbial community and chemical (e.g., C, N, P) vertical structure
486 was not evident within the first 6 cm of soil of coastal wetlands with elevated nutrient levels (e.g.
487 Lake Erie sites). The lack of vertical chemical gradients is unlikely to exclusively explain a
488 corresponding lack of vertical microbial community structure, as some wetland sites lower in
489 nutrient levels also did not experience vertical chemical gradients in this study (e.g. West Saginaw
490 Bay). One possibility is that a lack of vertical chemical structure in conjunction with high nutrient
491 levels in wetland soils could reduce vertical microbial community structure. It has been previously
492 demonstrated that concentrations of carbon electron donors may influence redox gradients within
493 wetland soils (Achtnich *et al*., 1995), and wetland microbial communities have been demonstrated
494 to correspond with soil redox gradients (Lüdemann *et al*., 2000; Edlund *et al*., 2008; Lipson *et al*.,
495 2015). However, connections between microbial community metabolic shifts with soil depth and
496 levels of dissolved organic carbon *in situ* remain unresolved in freshwater wetlands (Alewell *et*
497 *al*., 2008). Alternatively, another explanation for lack of vertical community structure could be
498 microsite heterogeneity throughout the soil matrix. Previous research in freshwater wetland soils
499 has suggested that microsite heterogeneity may explain coexistence of microbial functional guilds
500 (Alewell *et al*., 2008; Angle *et al*., 2017), which could substantially reduce vertical microbial
501 community structural gradients. However, it is necessary to better link microbial community
502 diversity, microbial activity, chemical structure, and microsite heterogeneity to establish
503 relationships between microbial communities and freshwater soil structure. As a caveat, it is
504 possible that chemical and microbial structuring still exists within wetlands with high nutrient
505 levels, yet is not evident within the first 6 cm of soil or at the spatial scale measured in this study.
506 Nevertheless, microbial communities within coastal wetlands with high nutrient levels did not
507 follow the same pattern of vertical structure evident in other comparable coastal wetlands, either
508 chemically or biologically, further suggesting that the microbial integrity of coastal wetland
509 systems may be susceptible to negative anthropogenic pressure.
510
511 While relationships between microbial diversity and nutrient levels among coastal
512 wetlands are strong, other unexplored variables unique to Lake Erie (such as geologic history)
513 could also be influencing uniqueness of chemical and microbial profiles in Lake Erie coastal
514 wetlands. The Lake Erie coastal wetland sites explored here were barrier (protected) wetlands,
515 while other wetland sites explored in this study are all classified as lacustrine (open water)
516 wetlands (www.greatlakeswetlands.org). As such, wave action from the Great Lakes impacted
517 wetlands within the western basin of Lake Erie to a lesser degree than other wetlands, thereby
518 reducing sediment export rates into the Great Lakes themselves. Hydrologic energy was found to
519 impact wetland primary productivity and respiration in Lake Huron coastal wetlands, suggesting
520 Great Lakes ecosystems may exert unique environmental forces on wetland microbial
521 communities (Cooper *et al.,* 2013). Low carbon export rates or elevated sedimentation rates may
522 exist in the western basin of Lake Erie as consequence of low wave action in these wetlands, which
523 may influence the chemical and biological structure (such as vertical microbial community
524 structure) within wetland soils of this region. Nevertheless, previous research at the same wetland

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locations explored in this study have demonstrated that wetlands within the western basin of Lake Erie are highly degraded with respect to other wetlands (Uzarski *et al*., 2017), particularly with respect to physicochemical conditions. Additionally, the same vegetation zone (dominated by cattails or bulrush) was sampled among all wetlands explored in this study as an attempt to reduce bias in distinct environmental conditions which may exist in other vegetation zones among wetland sites. Burton et al. (2002) suggested that soil organic content was related to plant zonation in Great Lakes coastal wetlands. Further research would be necessary to fully tease apart the effects of anthropogenic stress and other natural contributions to differences in microbial communities among coastal wetlands.

4.2 Relationships between microbial subnetworks and environmental gradients

Through network analyses, multiple subcommunities were delineated which were significantly related to environmental gradients (such as nutrients C, N, and P) among coastal wetlands sampled in this study. Specifically, a subnetwork of 69 microbial taxa was 93.8% predictive of nutrient level variation among coastal wetland soils. Several microbial taxa within this subcommunity were individually predictive of nutrient levels to a high degree, including several OTUs related to *Anaerolineaceae*, one OTU related to genus *Anaerolinea*, and another related to genus *Bellilinea*. From the genus *Anaerolinea*, two thermophilic chemoorganotrophs (*Anaerolinea thermophila* and *Anaerolinea thermolimosa*) have been isolated (Sekiguchi *et al*., 2003; Yamada *et al*., 2006). Only one isolated member has been established within the genus *Bellilinea* (*Bellilinea caldifistulae*); it has been described as a thermophilic, fermentative, obligate anaerobe which thrives in co-culture with methanogens (Yamada *et al*., 2007). It is unlikely that the OTUs found in our study are the same species as the isolated *Anaerolinea* and *Bellilinea* species, as coastal wetland soils are not high-temperature environments necessary for thermophilic species. Additionally, no OTUs related to methanogenic archaea were found within this subnetwork, suggesting that *Anaerolineacea* OTUs within coastal wetland soils may fluctuate independently of any specific methanogenic OTUs. It is possible that the *Bellilinea* OTU found within the subnetwork is related to nutrient level concentrations. This would support fermentative metabolism as noted within *Bellilinea caldifistulae*. It is important to note that several other studies have discovered OTUs related to *Anaerolineaceae* within wetland soils, with upwards of 90% relative abundance among *Chloroflexi* OTUs within these systems (Ansola *et al*., 2014; Deng *et al*., 2014; Hu *et al*., 2016). This suggests that there are probable mesophilic species yet to be isolated within this ubiquitous family of bacteria, which may be of high importance within wetland soils.

Betaproteobacteria were also found to significantly predict nutrient levels among coastal wetlands. Hu *et al*. (2016) found that both *Betaproteobacteria* and *Anaerolineae* were positively related to TN levels, which is consistent with the data presented here, and these two taxa were suggested to contribute to higher levels of heterotrophic activity. Further, *Anaerolineaceae* OTUs were consistently related to increasing C:N, suggesting that many taxa within this family have preference for recalcitrant carbon sources. As C:N also tends to increase with soil depth, it is also probable that the putatively obligate anaerobic *Anaerolineaceae* are coinciding with decreasing oxygen levels and/or changing metabolism requirements with increasing soil depth.

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571 Development of biological indices and establishment of indicator taxa have been suggested
572 as necessary for microbial communities within wetlands (Uzarski *et al*., 2017), particularly through
573 the use of high-throughput sequencing technologies which now allow for deep assessment of
574 microbial community composition and structure within environmental samples (Sims *et al*., 2013;
575 Urakawa & Bernhard, 2017). Specifically within Great Lakes coastal wetlands, it is integral to
576 develop ecosystem health indicators based upon multiple different groups of taxonomy, as separate
577 biological indices can present contrasting assessments of wetland health (Uzarski *et al*., 2017). As
578 microbial indicators have yet to be established in Great Lakes coastal wetlands, this research
579 begins the first steps in exploring how microbial communities can be used as an additional and
580 potentially important ecosystem health indicator. In addition to their importance as biological
581 signals for environmental health, microbial indicator taxa may play prominent roles in
582 bioremediation of excess nutrients and pollutants found within anthropogenically impacted coastal
583 wetlands. Network analyses in this study have allowed for the generation of hypothetical
584 subcommunities of diverse microbial taxa related to nutrient levels among Great Lakes coastal
585 wetlands, and could assist in further understanding of which microbial taxa may be responding to
586 anthropogenic stress in these ecosystems.
587
588 **5. Conclusions**
589
590 This study marks the first characterization of microbial communities within Great Lakes
591 coastal wetlands. Coastal wetlands are integral in the proper functioning of biogeochemical cycles
592 and environmental sustainability of the Great Lakes. While it has long been known that
593 anthropogenic pressure can impact animal and plant communities within these coastal wetlands,
594 this is the first evidence that these pressures may also be influencing microbial communities and
595 may be influencing biogeochemical cycles by extension. Alpha and beta diversity were both
596 related to nutrient gradients among and within regions, suggesting that variability in microbial
597 community structure is highly coupled to geochemistry within wetland soils. We propose that
598 wetland microbial community structure can also potentially be used to assess a wetland for
599 monitoring purposes. As illustrated within this study, wetland microbial community structure and
600 depth are decoupled within the wetlands experiencing the highest nutrient levels, likely originating
601 from terrestrial inputs due to human activity. As such, multivariate statistics (as used in the
602 methods of this study) may prove useful in examining relationships between wetland soil depth
603 and microbial community structure alongside microbial network analyses, which could provide
604 biological indicators of nutrient loading stress on coastal wetland habitats. We propose that
605 wetland microbial community structure can also potentially be used to assess a wetland for
606 monitoring purposes.
607
608 Further, this study provides insight on microbial community subnetworks and individual
609 OTUs, which were predictive of chemical concentrations, and may be useful for future
610 management of Great Lakes coastal wetland systems. Within subnetworks existed multiple taxa
611 with strong individual relationships to environmental gradients among coastal wetlands throughout
612 the Great Lakes. Even further, several community members within these subnetworks were
613 taxonomically related (such as OTUs related to *Anaerolineaceae* within *Chloroflexi*), suggesting
614 that specific taxonomic groups of microbes may be useful to explore further as potential biological
615 indicator groups. This study highlights the strength of network analyses (such as WGCNA) in

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616 delineating hypothetical networks of interacting microbes, and whether these networks are
617 predictive of physical or chemical gradients measured within an environment.
618
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627
628 **Author Contributions Statement**
629
630 DH processed samples, analyzed data, and wrote the manuscript. DU provided access to the
631 samples, aided sample selection, and statistical analysis. DL and KT provided guidance on
632 microbial analysis and KT, DU, and DL assisted in editing the manuscript.
633
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635
636 The authors declare no conflicts of interest.
637
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647
648 **Data Availability Statement**
649
650 Sequence data generated for this study can be found in the GenBank repository at
651 https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA417157. Other data generated for this
652 study, along with R code for replication of statistical methodology, can be found in the GitHub
653 repository at https://github.com/horto2dj/GLCW/.
654
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910 **Tables**

911

912 Table 1. Correlations between alpha diversity metrics and measured environmental variables.
913 Asterisks represent significance values where $p \leq 0.001$ (***), $p \leq 0.01$ (**), and $p \leq 0.05$ (*).
914

	Chao1	Shannon
P	0.31**	-0.09
S	0.42***	0.45***
NO_3^-	0.42***	0.24*
C:N	-0.03	-0.2
NUTR	0.24*	0.41***

915

916 Table 2. Pairwise perMANOVA results comparing pairwise differences between wetland regions
917 and differences between wetland soil depths. Values represent significant ($p \leq 0.01$) R^2 results,
918 and *n.s.* represents lack of significance ($p > 0.01$).
919

Region	BA	ESBT	LE	NSB	WSB
BA	-				
ESBT	*n.s.*	-			
LE	0.507	0.401	-		
NSB	*n.s.*	*n.s.*	0.524	-	
WSB	*n.s.*	*n.s.*	0.435	*n.s.*	-

Depth	Top	Middle	Bottom
Top	-		
Middle	**	-	
Bottom	**	*n.s.*	-

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922 **Figures**
923
924 Figure 1. Geographic map displaying location of sites sampled within this study. Colors of points
925 correspond to region sampled.
926
927 Figure 2. Principal Component Analysis (PCA) illustrating separation of samples based upon soil
928 geochemistry. Shapes and colors correspond to different wetland depths and regions, respectively,
929 as listed in the legend. Percentages on axes represent explained variance of that principal
930 component. Vectors represent impact of specific environmental variables on sample distribution.
931 NUTR represents OM values, which correlated significantly ($p \leq .01$, $r > 0.56$) to NO_3^-, OC, OM,
932 S, and TN. Ellipses represent 95% confidence intervals of region groupings.
933
934 Figure 3. Boxplot diagram comparing Chao1 diversity among wetland regions. Boxes with the
935 same letter are not significantly different, while those with no common letters are significantly
936 different ($p \leq 0.01$). Lines within boxes represent the median, hinges represent +/- 25% quartiles,
937 whiskers represent up to 1.5x the interquartile range. Colors represent wetland region.
938
939 Figure 4. Nonmetric Multidimensional Scaling (NMDS) plot illustrating separation of samples
940 based upon differences in microbial community structure. Shapes and colors correspond to
941 different depths and wetland regions, respectively, as listed in the legend. Vectors represent
942 correlations of environmental variables to the distribution of the microbial communities
943 represented in the plot.
944

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945 Figure 5. NMDS plots of each wetland region demonstrating separation of samples based upon
946 differences in microbial community structure, including **(A)** BA, **(B)** ESBT, **(C)** LE, **(D)** NSB, and
947 (E) WSB. Shapes and colors correspond to different depths and wetland sites, respectively, as
948 listed in the legends. Vectors represent correlations of environmental variables to the distribution
949 of microbial communities represented in the plots.
950
951 Figure 6. Network visualization and results of partial least squares analysis on the subnetwork
952 most correlated with NUTR. The y-axis represents correlation of OTU to OC values, whereas the
953 x-axis represents the node centrality. Points represent OTUs, and the color of points corresponds
954 to the phylum to which an OTU belongs. Point size corresponds to VIP score of that OTU. The
955 top 15 OTUs are labeled within the graph with corresponding lowest taxonomic identification
956 possible, and the level of that classification. D = Domain; P = Phylum, C = Class, O = Order, F =
957 Family, G = Genus.
958
959 Figure 7. Network visualization and results of partial least squares analysis on the subnetwork
960 most correlated with C:N. The y-axis represents correlation of OTU to C:N, whereas the x-axis
961 represents the node centrality. Points represent OTUs, and the color of points corresponds to the
962 phylum to which an OTU belongs. Point size corresponds to VIP score of that OTU. Only OTUs
963 with a VIP score > 1 were displayed for visualization purposes. The top 15 OTUs are labeled
964 within the graph with corresponding lowest taxonomic identification possible, and the level of that
965 classification. D = Domain; P = Phylum, C = Class, O = Order, F = Family, G = Genus.

Figure 1.JPEG

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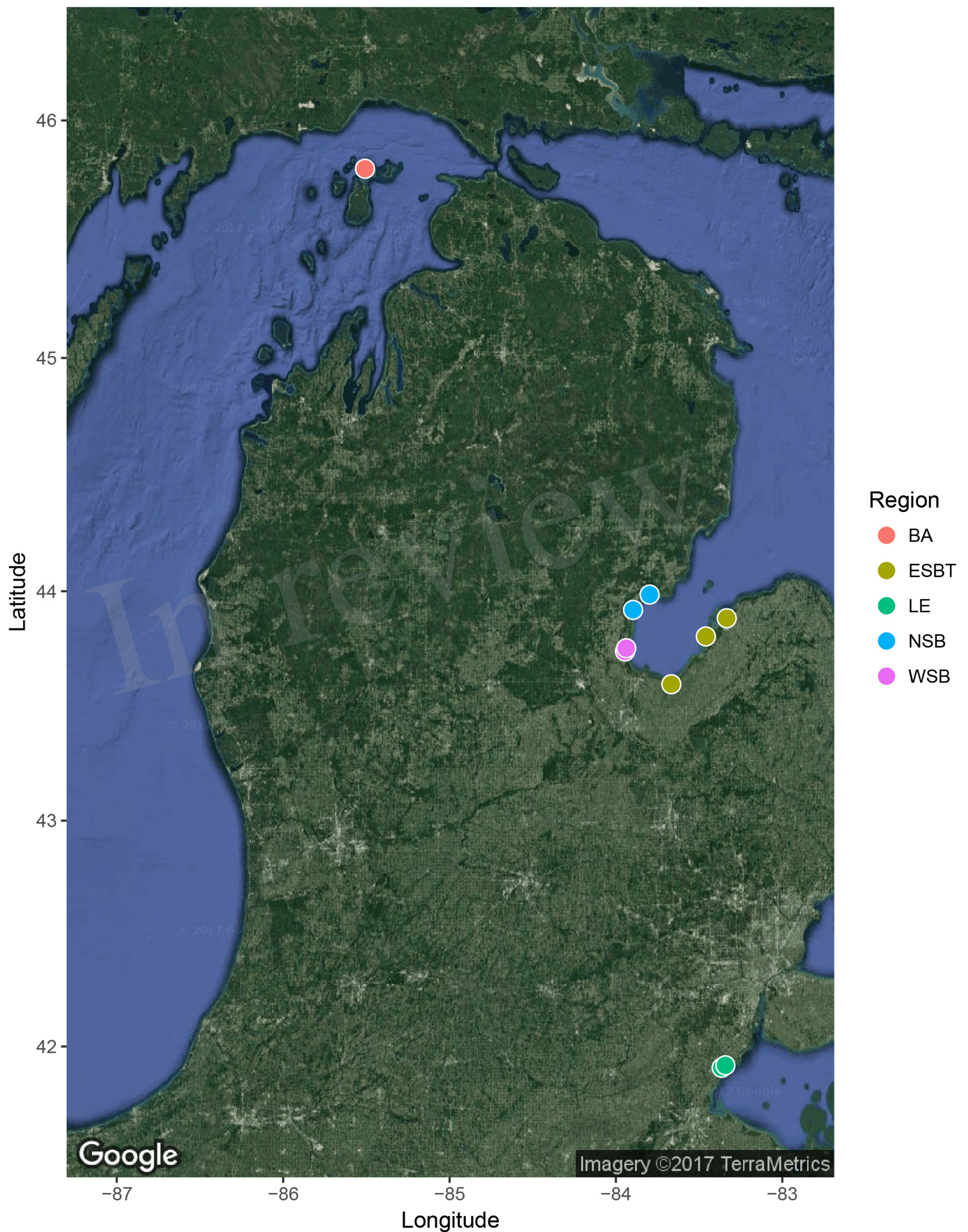

Figure 2.JPEG

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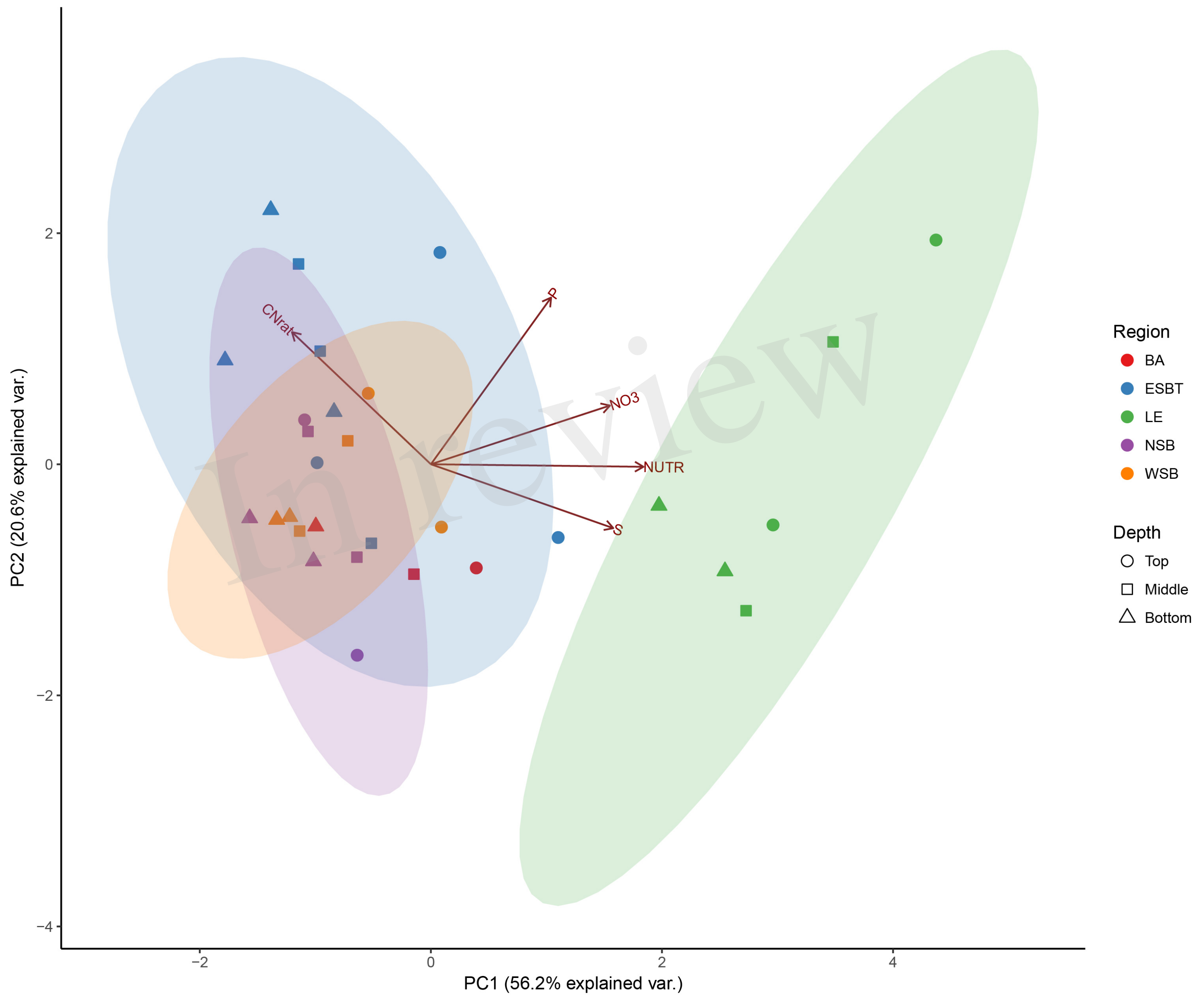

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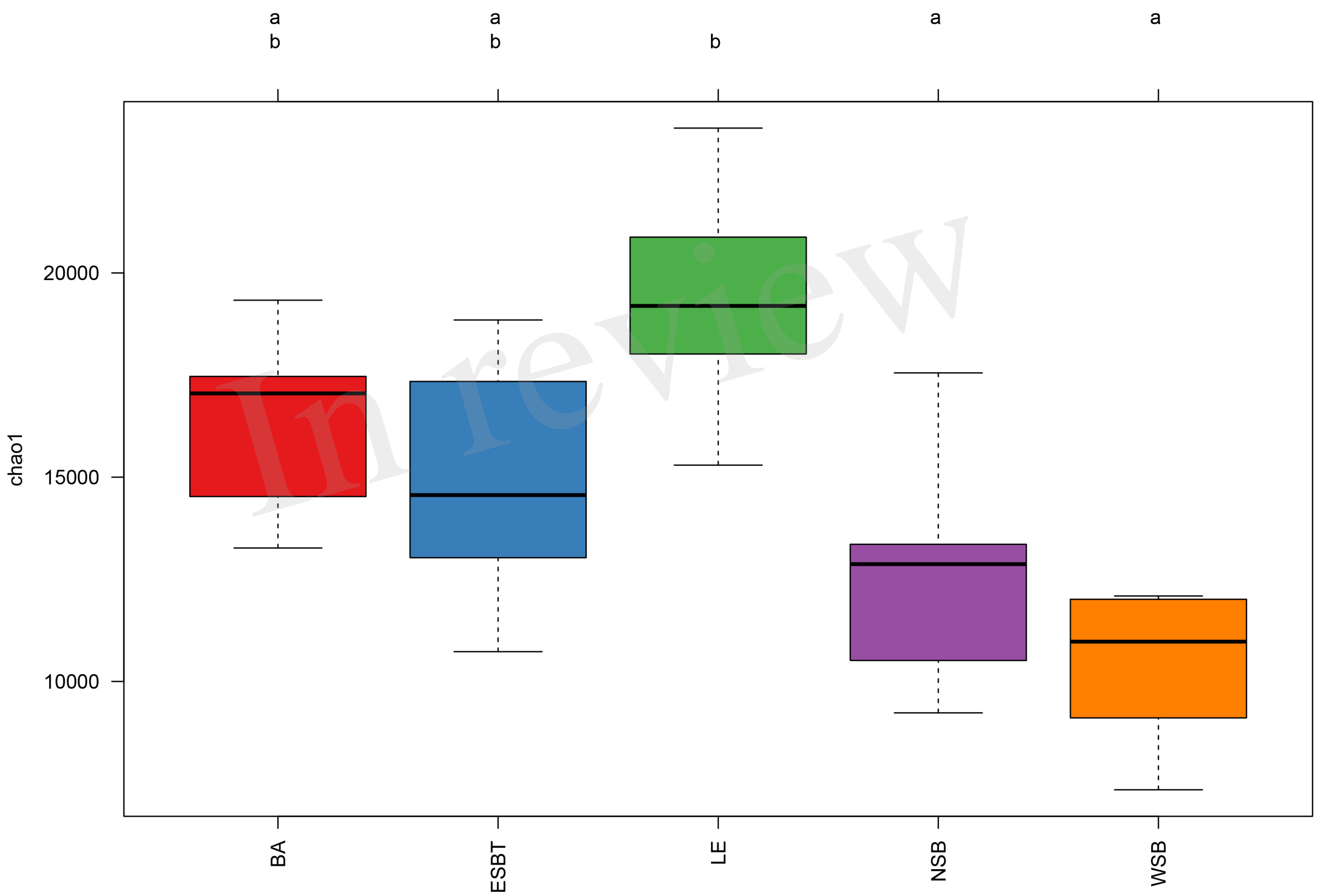

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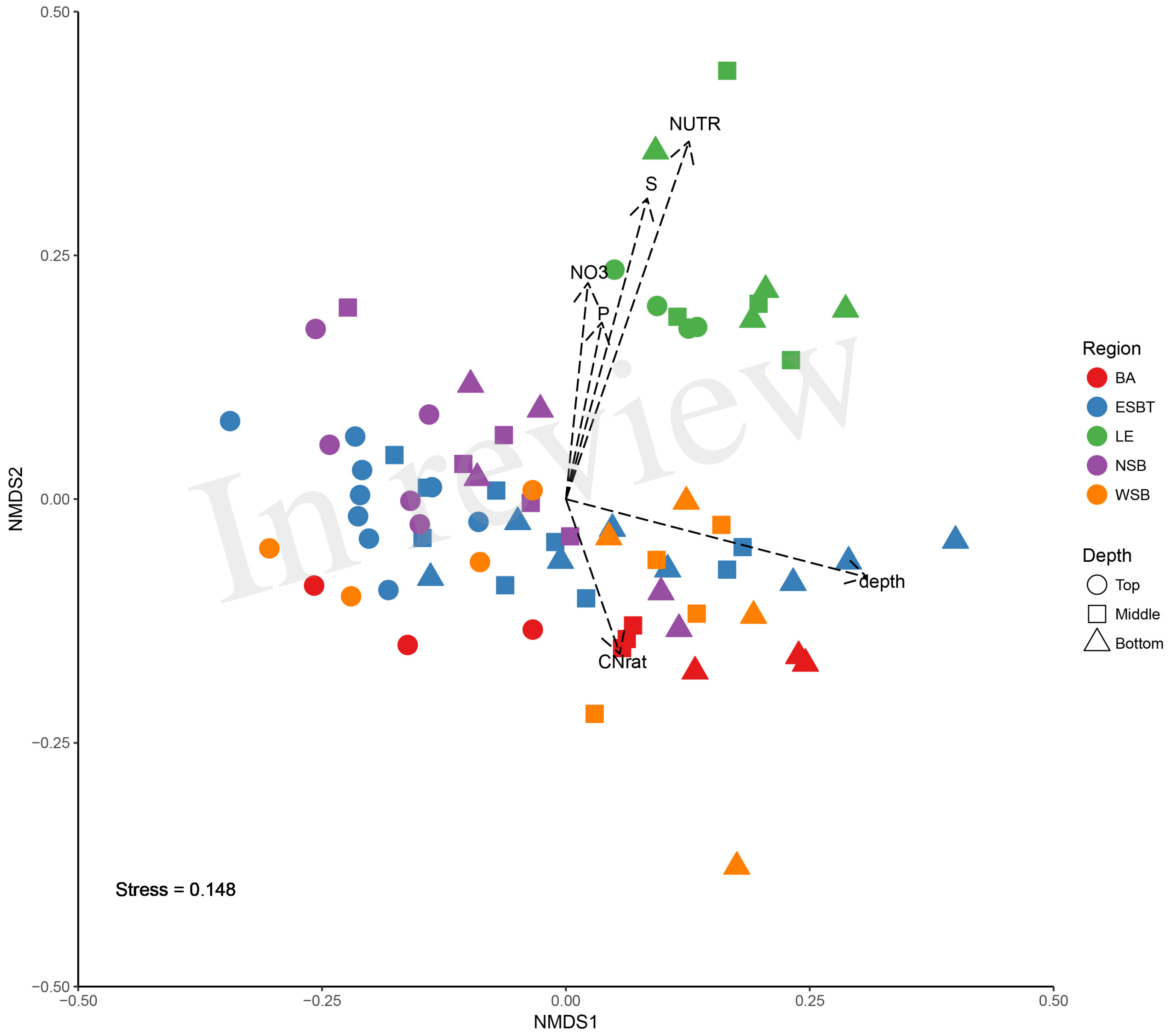

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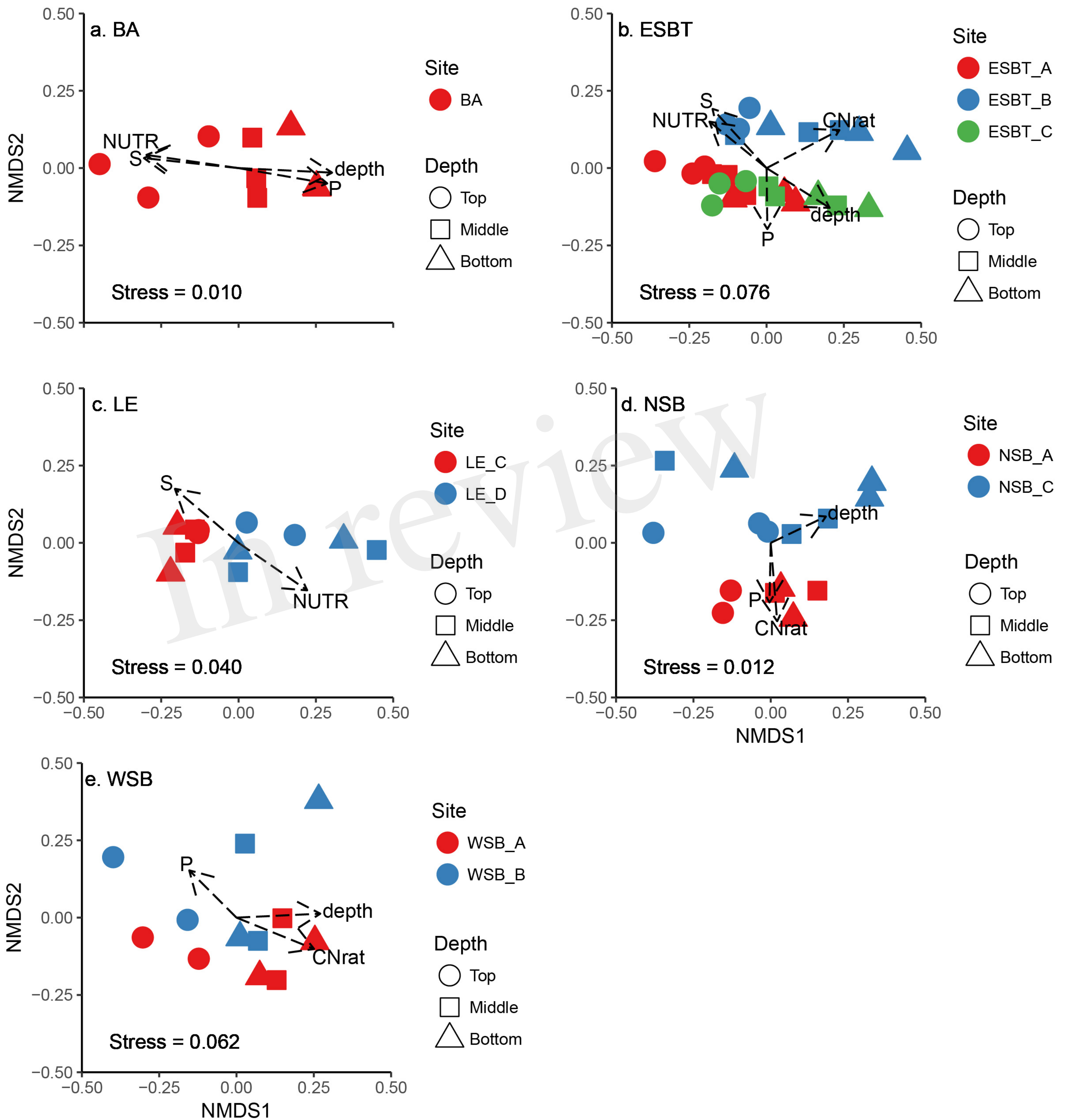

Figure 6.JPEG

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