

The microbiome of an amphibious plant shifts dramatically across the host life cycle.

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8 **Abstract**

9 Interactions between plants and their associated microbiomes are thought to enhance the capacity of
10 the host plant to overcome extreme conditions, yet the significance of microbially mediated stress
11 tolerance in most plant species and ecosystems remains unknown. For the first time, we examined the
12 microbiome of the amphibious plant *Eryngium castrense* (Apiaceae) inhabiting Mediterranean
13 climate ephemeral wetlands in California. *Eryngium castrense* has the capacity to survive as both an
14 aquatic and terrestrial plant, thereby living under contrasting extremes of water stress. Whether plant-
15 associated microbial communities are also affected by such changes, and what ecological role they
16 play while inhabiting amphibious plants, is an underexplored topic of plant-microbial interactions in
17 natural and artificial systems. We amplified and sequenced 16S rRNA genes from bacteria and
18 archaea to examine microbial communities associated with roots and shoots over the plants' full life
19 cycle. We observed that the microbiome changes from the aquatic stage to the terrestrial stage, and
20 that roots and shoots represent distinct habitats within the plant host ecosystem. When compared with
21 soil and water column samples, plant samples retained a unique, differentiated core microbiome.
22 Taxa located in the roots during the terrestrial stage were linked with potential functions such as
23 nitrogen acquisition, sulfur assimilation, and resistance to heavy metals, whereas aquatic roots held
24 potential phythoparasites. Overall, our results provide new insights into symbiotic relationships in
25 plants subject to stress-related to water saturation and deficiency.

26 **1 Introduction**

27 Symbiosis with microbes can improve plant health and habitat adaptation in natural and artificial
28 environments, with several plant traits co-regulated by the associated microbiome and the plant's
29 genome (Rodriguez, et al 2009; Morelli, et al 2020). Research focusing on symbiotic associations can
30 also enhance ecological theory and may even lead to the development of new biotechnology
31 (Redman et al, 2022). Among symbionts, microbial inhabitants of the rhizosphere and phyllosphere
32 are considered epiphytes, whereas microbes residing within plant tissues (the endosphere) are
33 considered endophytes. These different types of symbionts can also occupy different within-plant
34 habitats (compartments)—such as roots, shoots, and leaves—establishing beneficial associations with
35 their host plants (Carrell & Frank, 2014; Fan, et al 2020). Selection imposed by plant compartments
36 strongly shapes the diversity and composition of the microbiota. Different plant tissues such as
37 leaves, roots, or flowers can harbor unique taxa, modulating microbe-microbe interactions, due to

38 both their structural differences and exposure to soil versus air and feeding back to host fitness
39 (Fitzpatrick et al, 2020). Nevertheless, sometimes bacterial communities in leaves and roots can be
40 surprisingly similar (Bai et al, 2015; Van der Heijden et al, 2016). While plant microbiomes are
41 usually dominated by Proteobacteria (particularly those of the α and β classes), and other major
42 groups include Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Verrucomicrobia, and
43 Acidobacteria (Turner et al, 2013), they can be variable and complex. In addition, existing evidence
44 suggests that variation in the abiotic and biotic environment can also exert direct and indirect effects
45 on plant-associated microbiota; climate-driven geographical variation can drive the composition of a
46 microbiome (Oono, et al 2017; Harrison & Griffin, 2019). The plant microbiome therefore holds
47 interesting parallels to other environmental systems and their microbiomes—for instance, the gut
48 microbiome or the Earth microbiome—with conceptually similar questions regarding horizontal
49 transfer of symbionts, diversity drivers, and influence in host niche expansion and plasticity (Redman
50 et al, 2011; Redman et al, 2022). This is of importance in a global change context, for example, as
51 global temperature increases and variation in both abiotic and biotic factors can indirectly shape plant
52 microbiota through plant responses (Suryanarayanan & Shaanker, 2021; Ware, et al 2021). Given the
53 known importance of plant microbiomes and their variations, examining the communities present on
54 and within different and unique plant species has the potential to provide new insights.

55 Vernal pools—ephemeral bodies of water—provide habitat for specialized plants and animals, and
56 represent an extreme version of the climatic variation characteristic of many other ecosystems. With
57 seasonal water saturation during winter and gradual desiccation conditions the rest of the year, vernal
58 pools (en español: *charcas vernaes*) trigger evolutionary processes of dormancy and amphibious life
59 cycles with unique morphological traits. Many plant lineages have independently adapted to vernal
60 pools conditions, including aquatic plants that have adapted to desiccation conditions (e.g. *Isoetes*
61 *howellii*), and plants whose ancestors were terrestrial taxa that became aquatic (e.g. *Eryngium spp.*).
62 Typically the aquatic morphology, ‘grass-like tufts’, is emblematic of the aquatic period of the pools
63 in winter and early springtime. Later in the season, after evaporation and total desiccation of pools,
64 plants acquire a terrestrial morphology that is spiny and resembles more woody herbaceous plants
65 (Keeley, 1999). However, little is known about the microbiomes of these fascinating plants.

66 Interactions between plants and their associated microbes can provide higher fitness under extreme
67 environmental conditions (Baedke, et al 2020; Trivedi et al, 2020) and in the context of future of
68 agriculture practices (i.e. global change, artificial life support systems), achieving the ability to assist
69 plants in rapidly adapting or acclimating signifies a milestone in the development of resilient crops.
70 In this vein, amphibious plants, inhabiting ephemeral wetlands such as vernal pools are ideal study
71 cases, as these plants tolerate wide extremes of water availability and other abiotic stress. These
72 plants and their associated microbial symbionts can provide key insights into stress tolerance. To our
73 knowledge, this study is the first of its kind in exploring amphibious plants and their associated
74 microbiome. We examined *Eryngium castrense* (Apiaceae), colloquially known as the Great Valley
75 button celery (Figure 1). This plant species is endemic to the California Central Valley, with several
76 amphibious members of this genus in other regions of California, USA, and Baja California, Mexico
77 in other vernal pool complexes.

78 In this present study, we investigated the dynamics of the microbiome (specifically bacterial
79 endophytes) within the amphibious vernal pool plant *E. castrense*. Our first objective was to compare
80 the differences in endophytic community diversity and composition between the aquatic and
81 terrestrial stages of these plants. We hypothesized that if the microbial community plays a role in
82 plant stress tolerance during extreme stages, we would observe shifts in the community composition
83 and diversity aligned with aquatic and desiccation stages. Additionally, we examined the

84 differentiation within the host habitat by comparing the root and shoot compartments. We aimed to
85 determine whether these plant compartments harbor distinct microbial communities, reflecting the
86 different above-ground and below-ground needs of the plant. We also examined community
87 assembly at the ecosystem level, focusing on differences in composition and diversity between
88 endophytic communities and immediate plant surroundings, including the soil surrounding the roots
89 and the water column that is in contact with the shoots. Our objective was to identify a unique core
90 microbiome in the soil, water, and plants, indicating the presence of specific ecological niches.
91 Finally, we investigated the influence of the surrounding environment as a filtering agent for
92 community composition. We aimed to understand the extent to which the microbiome of amphibious
93 plants is shaped by the plant host, environmental selection, or dispersal, considering the influence of
94 landscape heterogeneity. Our overarching hypothesis motivating this work was that the microbial
95 community is relevant to plant stress tolerance across the extreme stages in vernal pools, with
96 microbes supporting critical functions when the plant host is subject to desiccation and water
97 saturation stresses. We anticipated that compartmentalization within the plant, based on tissue
98 differentiation, would result in niche specificity, with distinct microbial functions within shoots and
99 roots. Additionally, we expected that landscape properties would influence the composition of the
100 plant microbiome.

101 2 Methods

102 1.1 Study area

103 The study area is located in the heart of the San Joaquin Valley, California, USA, a 400 kilometers
104 long and 80 kilometers wide open are, flanked on the east by the Sierra Nevada and on the west by
105 the Coast Range mountains. The climate of the region is Mediterranean, characterized by cool, wet
106 winters and hot, dry summers; annual rainfall generally ranges from 230-380 mm with 90% of the
107 precipitation occurring from November to April. The western and eastern boundaries of the region
108 delimit a distinct topographic and biogeographic unit of undulating terrain, from above the historic
109 San Joaquin River floodplain, to the base of the Sierra Nevada foothills. This area supports the
110 largest block of unfragmented vernal pool habitat remaining in California, characterized by low-slope
111 basins with undulating *mima mound* topography that typically supports a high density of vernal pools
112 (Vollmar, 2002). The sampling was performed at the University of California, Merced Vernal Pools
113 and Grassland Reserve (MVPGR) (Figure 2), adjacent to the main campus of the University of
114 California, Merced. The MVPGR is tightly managed for conservation. However, we hypothesized
115 that development effects may be stronger on the west side of the reserve facing campus and the city
116 of Merced. On the western boundary of the reserve, activities derived from land management, such as
117 high cattle concentration, disturbances derived from urbanization, such as concrete dust, light
118 pollution, noise or, activities derived from the UC campus such as visitors, trash, and motor vehicles,
119 may lead to alterations of the natural landscape and create an anthropogenic impact zone.

120 1.2 Sampling

121 During the first months of life development, *Eryngium castrense* resembles aquatic grasses
122 (“isoetoid” morphology, Fig 1a), extended above the vernal pool water surface. After the pool dries
123 out, it becomes weedy, with sharp, spiny leaves (Fig 1b), flowering throughout the hot summer
124 (Preston, et al 2023). This plant species has well-differentiated roots and shoots, and a differentiated
125 phenotype as a result of the metamorphosis from aquatic to terrestrial morphology, corresponding to
126 soil flooding and desiccation. *E. castrense* is a glabrous, ascending herbaceous plant, multi-branched
127 and spiny, with lanceolate, deeply pinnate leaves (Baldwin & Goldman, 2012; Preston, et al 2023).

128 This plant species is abundant inside the limits of UC Merced's Vernal pools and grassland reserve
129 and many vernal pools in California's Central Valley.

130 Collections of specimens of *E. castrense* were made at five vernal pools (Table 1). Sites were
131 selected by following a five-kilometer transect, starting from the western boundary of the reserve,
132 adjacent to the UC Merced campus, moving east, each site one kilometer apart. Collections were
133 made in two seasons; during the aquatic period, which occurred in winter 2018 and comprised 20
134 plant specimens (four individuals per pool); followed by a collection of 25 specimens (five
135 individuals per pool) in late spring 2018, for a total of 45 plant specimens. This sampling comprised
136 the aquatic root and the shoot during wintertime (inundation of the basin of the vernal pool), and the
137 terrestrial root and shoot during springtime, respectively, therefore, a final number of 90 tissue
138 samples were used. In addition to plant tissue samples per vernal pools, parallel collections of soil
139 and water were included in the study to obtain a panoramic view of the microbial dynamics across
140 the ecosystem layers, from soil, to water column and plant tissues.

141 **1.3 Sterilization of tissues and library preparation**

142 Sterilization was performed at the laboratory following a mechanical procedure: two rinses with
143 distilled water for two minutes each, to separate the soil aggregates from the biological sample. The
144 following rinses were then used: ethanol 75%, one rinse for one minute; Milli Q water, one rinse for
145 two minutes; bleach 5%, once rinse for one minute; and Milli Q water, one rinse for five minutes
146 (Guzman, et al 2020). After the sterilization, roots and shoots were separated with an autoclaved
147 blade, fast-frozen by liquid nitrogen, and pulverized using a mortar. From the pulverized plant tissue,
148 0.2g was measured and processed with a Qiagen PowerSoil[®] DNA extraction kit. A total of 95
149 extracted DNA aliquots were obtained from tissues for sequencing the 16S rRNA gene. In separate,
150 soil samples and water samples collected to compare with plant tissue samples, were processed
151 following the protocol in Montiel-Molina, et al. (2021), for a total of 10 extracted DNA aliquots, 5
152 from soil samples and 5 from water samples.

153 Prior to PCR amplification and library preparation, extracted DNA aliquots were analyzed for
154 concentration and purity via Qbit[®] technology. Peptide nucleic acid (PNA) clamps to prevent the
155 amplification of plant mitochondria and chloroplast DNA were added to each PCR master mix
156 (Lundberg et al, 2013). A 50ul PCR mix was prepared for each sample as follows: water 31.8 ul;
157 Buffer 1x 31.8ul; dNTP(0.2uM) 1ul; 926R_Adapter (0.4uM) 2ul; PNA chloroplast (1.2uM) 0.6ul;
158 PNA mitochondria (1.2uM) 0.6ul; BSA (0.2 mg/ml) 1ul; Taq(0.1 U/ul) 1ul; Barcode primers (10uM)
159 2ul; DNA template 5ul. Barcoded primers targeting the 16s rRNA V4-V6 coding regions
160 (515F_915R) were used for the analysis of the bacterial endophytes (Quince, et al 2011; Apprill, et al
161 2015; Parada, et al 2016). The thermocycler was programmed as follows: 94°C for 3 min; 35 cycles
162 at 94°C for 45 sec; 78°C for 10 sec; 50°C for 30 sec; 72°C for 1 min; 72°C for 10 min and then held
163 at 4°C. Final aliquots were analyzed for DNA concentrations using a Qbit[®] and pooled together for
164 purification. Sequencing was achieved on the Illumina MiSeq[®] at UC Davis Genome Center. Soil
165 and water samples were prepared and analyzed at Argonne National Lab, Chicago.

166 **1.4 Bioinformatics and diversity analysis**

167 The platform Qiime2-v.2022.2 (Bolyen et al, 2019) was used for microbiome bioinformatics analyses
168 of plant tissues, soil and water samples, and preliminary microbiome analysis. Sequence reads were
169 processed with DADA2 after demultiplexing, to filter chimeric reads from the analysis and to infer
170 amplicon sequence variants (ASVs) (Callahan et al, 2016). The SILVA database was used to assign

171 taxonomic ranks (Quast et al, 2013). Chloroplast and mitochondria sequences were filtered from all
172 analyses. Low sequence reads were found in terrestrial samples (shoots) from the sites KM4 and
173 KM5, and these samples were removed from further analysis.

174 For plant samples we assessed diversity across vernal pool compartments, roots and shoots and plant
175 host morphology (aquatic and terrestrial, water and soils samples. Analysis was made with R studio
176 (v.1.3.1093) using the package Qiime2R-v0.99.1 (Bisanz, 2018) to manage artifacts produced with
177 Qiime2. Diversity and community composition analysis, comprising alpha diversity, relative
178 abundances, distance metrics (Bray-Curtis, Unifrac), and dissimilarity analysis between samples
179 (PCA, ADONIS-PERMANOVA) were performed using Qiime2-v2022.2 (Bolyen et al, 2019),
180 Phyloseq-v1.36.0 (McMurdie & Holmes, 2013) and Microbiome-v1.14.0 (Lahti et al, 2017). Pearson
181 correlations and Mantel tests were performed with Vegan 2.6-4 (Oksanen et al, 2022), to assess the
182 effect of environmental properties, the distance between sampling sites and anthropogenic
183 disturbance on microbial community composition. Heatmaps for abundant taxa within tissue samples
184 were produced with Phyloseq-v1.36.0 (McMurdie & Holmes, 2013). We blasted sequences from the
185 top 30 ASVs against the NCBI database to determine their identity and potential function.

186 Analysis of the core microbiome of roots were considered given data robustness in diversity analysis.
187 Data from plant roots samples and additional soil and water samples were processed with the R
188 package Microbiome-v1.14.0 (Lahti et al, 2017), and graphical representations were made with the
189 Venn diagram in Euler-v7.2.2 (Larsson, 2022). The core microbiome was defined as taxa present in
190 least 90% of the samples. Core microbiome sequences were blasted against the NCBI database.

191 **2 Results**

192 We observed clear differences in the diversity of microbial communities across the plant life cycle
193 (aquatic and terrestrial) and plant compartments (root and shoot). Proteobacteria and Bacteroidetes
194 taxa dominated the community of microbial endophytes across both plant life cycle and
195 compartments, but with multiple patterns in community dynamics (see below). Samples varied
196 greatly in the number of ASVs present, with rarefaction curves reaching asymptotes at a sampling
197 depth below 1000 sequences (Supplementary Fig. 1).

198 **2.1 Representative taxa in amphibious plants**

199 Samples varied substantially in taxa composition. At the phylum level, Bacteroidetes and
200 Proteobacteria dominated the microbiome of *E. castranse*, followed by Acidobacteria,
201 Cyanobacteria, Firmicutes, Verrucomicrobia and Tenericutes (Fig 3). The classes
202 Alphaproteobacteria, Betaproteobacteria, Flavobacteria, Gammaproteobacteria, Saprospirae, and
203 Sphingobacteria were the most abundant across plant compartments. Taxa from the phylum
204 Bacteroidetes were present mostly in roots and we observed an evident shift in taxa abundance
205 between the aquatic stage and terrestrial stage. Within this phylum, Chitinophagaceae,
206 Cytophagaceae, Flavobacteriaceae and Sphingobacteriaceae families were represented in roots
207 samples, and Weeksellaceae was only present in roots samples of the terrestrial morphological stage.
208 Proteobacteria were represented by Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria,
209 Epsilonproteobacteria, and Gammaproteobacteria; roots harbored the most taxa in these classes in
210 aquatic and terrestrial samples. Firmicutes and Actinobacteria were mostly present in terrestrial roots,
211 whereas phyla such as Verrucomicrobia and Tenericutes were present in all plant samples. Firmicutes
212 of the class Clostridia were detected mostly in samples from roots in the aquatic stage, with taxa in
213 the family Veillonellaceae, Clostridiaceae, Bacillaceae, Peptostreptococcaceae and
214 Lachnospiraceae. Taxa in the phylum Acidobacteria belonged to the families Bryobacteraceae,

215 Acidobacteriaceae, Holophagacea, Koribacteraceae, Solibacteraceae, Ellin6075, MV65 and OPB3,
216 mostly present in samples representing the aquatic stage in roots, and absent in shoots; in contrast
217 terrestrial stage only allocated taxa in the family Acidobacteriaceae. We detected unknown taxa from
218 the phylum Cyanobacteria mostly in shoots samples of the terrestrial stage. Taxa from the
219 Phormidiaceae family were detected in shoots of the aquatic phase and taxa from the family
220 Pseudanabaenaceae were detected in aquatic roots.

221 **2.2 Microbial endophytic communities across plant compartments and plant morphology.**

222 Community dissimilarity among samples was analyzed using Unweighted-Unifrac and Bray-Curtis
223 metrics, and the significance of sample clustering was corroborated by the ordination method
224 Principal Component Analysis (Fig. 4) and PERMANOVA-ADONIS (Table 2). The samples were
225 best defined by aquatic and terrestrial morphological stages of the plant specimens, followed by roots
226 and shoots. The ordination analysis resulted in the formation of distinct clusters. One cluster
227 consisted of samples corresponding to roots in the terrestrial morphological stage, while another
228 cluster consisted of samples with roots in the aquatic morphological stage. Additionally, there was a
229 cluster composed of samples defined by shoots in the terrestrial morphology, and a diffuse cluster
230 formed by shoots in the aquatic morphological stage. PERMANOVA-ADONIS of Bray-Curtis and
231 Unweighted-Unifrac dissimilarity indexes validated a significant differentiation between plant
232 compartments and morphological stages (Table 2).

233 Alpha diversity, as measured by richness, Shannon index, Simpson's index, ACE (abundance-based
234 coverage estimators), and Simpson's evenness, also displayed differences between roots and shoots
235 and between terrestrial and aquatic morphological stages. Root samples in both aquatic and terrestrial
236 stages had higher diversity in contrast with shoots (Fig. 5). Aquatic stages showed higher overall
237 diversity than terrestrial stages. Based on the Kruskal-Wallis test, the difference in diversity between
238 sample types was significant (Table 3).

239 The 30 most abundant sequences (Fig. 6) displayed varying percent sequence identity compared with
240 taxa in the NCBI database, nevertheless in ecological terms, the composition reflected consistent
241 niche differentiation according to plant compartment and between terrestrial and aquatic stages. For
242 instance, Cytophagaceae spp, Chitinophaga spp, Oxalobacteraceae spp, Flavobacterium frigidarium,
243 *Chitinophagia arvencicola*, *Cryseobacterium aquaticum*, *Pantoea agglomerans*, *Niastella soli* among
244 a few other taxa within the families Mucilaginibacter, and Chitinophagia showed higher relative
245 abundance in root tissues in both aquatic and terrestrial stages. Shoots harbored fewer of these taxa,
246 however, terrestrial shoots had relatively high abundances of *Pantoea agglomerans*, *Xanthomonas*
247 *translucens*, and *Uzinura diaspadicola* (Fig. 6).

248 **Impact of water, soil, environmental factors, and geographic distance on the plant microbiome**

249 In contrast to the significant influence of plant compartments and plant morphology over the
250 endophytic community, Pearson correlations showed no significant influence from the landscape
251 spatial distances, anthropogenic activities, or soil properties. The Mantel test showed no significant
252 distance-decay relationship between plant sample community dissimilarity and geographical distance
253 between sites (supplementary Table 1).

254 Comparisons between communities in the plant endosphere and those in the surrounding water and
255 soil environment showed differentiation. Beta diversity analysis showed habitat differentiation
256 between samples of aquatic root morphology, terrestrial root morphology, soil and water from vernal
257 pools ecosystems (Supplementary Fig. 2). We analyzed the core microbiome from each of these

258 habitats; aquatic and terrestrial root tissues, water and soil (Table 4). Twenty one taxa represented the
259 core microbiome in soil samples, and zero taxa in the case of water samples; six taxa represented the
260 root endosphere (including aquatic and terrestrial stages), two taxa specifically belong to aquatic
261 roots and three taxa belong to terrestrial roots, and only one taxon was shared between aquatic and
262 terrestrial roots (Fig. 7).

263 **3 Discussion**

264 Plant species with an amphibious lifestyle are an interesting subject of study given their adaptations
265 to contrasting conditions. These amphibious plants are mostly restricted to ephemeral wetlands
266 known as vernal pools in Mediterranean climate regimes (Keeley and Zedler, 1998), where they have
267 adapted to water deficit, which for some species has led to differentiated morphologies throughout
268 their life cycle (Keeley, 1999). For this study, we aimed to obtain novel information from a plant-
269 microbe symbiosis perspective by providing the first description of the microbial endophytes in
270 amphibious plants and addressing the transitions during their life cycle. Information on microbial
271 community assembly may be key to understanding the mechanism behind the amphibious behavior,
272 as microbial symbionts could be involved in alleviating plant stress under desiccation conditions,
273 with important implications for both agricultural and natural ecosystems where water becomes a
274 limiting factor.

275 When vernal pool plants are young, they resemble an aquatic grass, and as they become mature their
276 morphology is more similar to a spiny weed. We studied the dynamics of the endophytic community
277 across the plant life cycle as a first step towards elucidating the potential role of microbes in
278 alleviating the stress triggered by the inundation and conditions experienced by the plants. The
279 results obtained from the beta diversity metrics defined two well-delimited niches according to
280 aquatic and terrestrial stages and the morphology of the amphibious plants. Our result supported the
281 hypothesis that the endophytic community varies by plant stage, and across the life cycle of the
282 plant. Our results are consistent with Zhou et al (2023), who reported significant differences in the
283 microbiome of young and mature specimens of *Suaeda salsa*, a plant inhabiting high salt
284 concentration habitats. Our study yielded contrasting results regarding water stress compared to other
285 studies—but this is likely due to the more pronounced differences in water availability captured in
286 our study compared to the more limited differences examined previously. For example, in Eucalyptus
287 trees, water deficit treatments were not a significant driver of microbial community variation
288 (Dasgupta et al, 2020), and a study of pepper plants roots and fruits microbiomes reported that
289 bacterial endophytes were not affected by water irrigation limitation (Cui, et al 2020). Both studies
290 suggested that endophytic communities were resilient to water stress. Yet, in our study, plant stage
291 (aquatic vs terrestrial) was a stronger driver of microbial community variation than compartment,
292 supporting our hypothesis that endophytic communities of vernal pool plants shift significantly with
293 the extremes that plants experience in vernal pool ecosystems. This may suggest a role for bacterial
294 endophytes in alleviating part of the stress generated by the water deficit and inundation conditions
295 within vernal pools. This result also suggests that amphibious plants have the capacity to select their
296 symbionts. However, inoculation experiments will be required to test if recruitment of symbiotic
297 microbial communities is part of vernal pool plant adaptation to the contrasting conditions of
298 inundation and desiccation.

299
300 Differences in microbial communities between compartments is generally attributed to the presence
301 of differential bioactive compounds. For example, Toju et al. (2019) suggested that in tomato plants,
302 alkaloids, caffeic acid derivatives, polysaccharides and alkenes affect the microbiome. We did not
303 analyze secondary metabolites in our study, but the taxa associated with above- and below-ground

304 provide an approach to understand ecological relations (Table 5). Other reasons can include
305 unhealthy conditions (with dark green leaves, signs of grazing) that diminish the diversity in the
306 tissues (Yang et al, 2022). Finally, the niche differentiation can be also attributed by the lack of free
307 entry of endophytes into living tissues in roots and shoots, which might require bacterial capabilities
308 to hydrolyse the hydrophobic incrustations of the walls of epidermal, hypodermal, endodermal and
309 other cortical cells (McCulley, 2001).

310 The microbial endophytic communities in our study were dominated by Bacteroidetes and
311 Proteobacteria (Fig. 3), comparable to the endophytes in Eucalyptus, which require large amounts of
312 water (Dasgupta et al, 2020) and aquatic habitats defined by low tides and high oxygen availability
313 (Stevens et al, 2005). The Proteobacteria phylum is ubiquitous in both aquatic and terrestrial
314 ecosystems, and endophytic taxa from this phylum fill important niches in relation with
315 biogeochemical cycles such as nitrogen (Zhou et al, 2020, Bai et al 2022). Bacteroidetes are likewise
316 involved in a variety of processes, including pathogen suppression (Lidbury et al, 2020). In our
317 study, we observed taxa related with phytopathogens (although no symptoms of disease were noticed
318 at the moment of handling specimens) and nitrogen fixers in roots during the aquatic stage of the
319 plants, similar to rice crops (Verma et al, 2001). We summarize in Table 5 information about the top
320 ASVs of endophytes to provide insight into their potential ecological role in amphibious plants.

321 Beyond niche differentiation by plant compartment, we examined the surrounding soil and water to
322 compare microbiomes between these ecosystemic layers. These ecosystem compartments (soil, water
323 and plant tissues) showed strong dissimilarities in bacterial community composition. Other studies
324 have reported that endosphere, rhizosphere and soil hold a niche differentiation, and a transition from
325 soil, rhizosphere, rhizoplane and plant endosphere, where diversity is higher outside the plant tissues
326 (Wang et al, 2022). In our study, soil samples exhibited higher microbial diversity in comparison
327 with plant tissues (supplementary Figure 3), and we detected a similar pattern when tissues were
328 compared with water samples, however these samples were more variable than soil.

329 **4 Conclusion**

330 In Mediterranean ecosystems where summer is characterized by desiccation and winter by
331 inundation, amphibious plants specially adapted to conditions of stress are hypothesized to have
332 microbial symbionts with the potential to alleviate water stress. This study shows that in the
333 amphibious vernal pool plant *E. castranse*, plant host morphology and tissue compartmentalization
334 has strong influence over the bacterial endophytic community assembly. Future research on microbial
335 endophytes in amphibious plants should incorporate the assessment of the fungal endophytes as well,
336 as fungi play a significant role in the plant microbiome and have unique ecological functions.

337 This pioneering study on the microbiome of vernal pool amphibious plants provides preliminary
338 findings about the role of endophytes in ecosystems subject to environmental change events, with
339 parallels in other systems in our changing world. The interactions and mechanics underlying this
340 microbial-plant association is complex, with endophytic communities also occurring during periods
341 of variation in oxygen concentrations and other effects within the environment not examined here.
342 Future research should incorporate those other conditions, which have effects in other ecosystems
343 and with other organisms. Presently, there is a growing interest in artificial environments that are
344 resilient and have a circular energy flow. The microbiome of amphibious plants can be an interesting
345 addition to research focusing on such technology. We propose that future research on the benefit of
346 endophytes to amphibious plants involve an experimental approach, where plants are inoculated with
347 endophytes and tested for survival in growing chambers, and we suggest the use of specimens within
348 the same family plants that are used as crops as we did here, using *E. castranse*, a member of the

349 carrot family. The importance of these following steps will be useful for the implementation of
350 agricultural systems.

351 We suggest exploring plant relatives from the carrot family for further studies, as the data obtained in
352 this study can serve as a foundation for future research involving crop plants from the same family.

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552 6 Tables

553 **Table 1.** Sampling sites at Merced Vernal Pools and Grassland Reserve (MVPGR). Each site belongs
554 to a single vernal pool, with a distance of one kilometer from each other. Three soil types are
555 represented: Corning, Redding, and Hopeton.
556

<u>Collection ID</u>	<u>Site ID</u>	<u>Distance from urbanization</u>	<u>Soil parent material</u>	<u>Longitude</u>	<u>Latitude</u>
MONT-0VP4	K0	0 Kilometers	Corning	-120.42011	37.3788671
MONT-1VP4	K1	1 Kilometer	Redding	-120.41254	37.33804231
MONT-2VP2	K2	2 Kilometers	Redding	-120.40515	37.3878441
MONT-3VP1	K3	3 Kilometers	Corning	-120.38972	37.3898201
MONT-4VP5	K4	4 Kilometers	Corning	-120.37992	37.3889871
MONT-5VP1	K5	5 Kilometers	Hopeton	-120.376	37.3927671

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563 **Table 2.** PERMANOVA-ADONIS based on microbial community composition of aquatic roots,
 564 aquatic shoots, terrestrial shoots, and terrestrial roots.

Permanova-ADONIS sample dissimilarity significance

<i>Dissimilarity method</i>	<i>Groups</i>	<i>SumsOfSq</i>	<i>MeanSqs</i>	<i>F.Model</i>	<i>R²</i>	<i>Pr(>F)</i>
Plant compartment Unw-Unifrac	2	1.07	1.07	4.37	0.08	0.001
Morphological stage Unw-Unifrac	2	1.19	1.19	4.91	0.08	0.001
Plant compartment Bray-Curtis	2	2.66	2.66	6.13	0.06	0.001
Morphological stage Bray-Curtis	2	1.17	1.17	2.60	0.02	0.001

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566 **Table 3.** Kruskal-Wallis non parametric analysis on alpha diversity metrics between roots and
 567 shoots samples, aquatic and terrestrial morphological stages.
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<i>Kruskal-Wallis Analysis</i>	<i>Shannon</i>	<i>Simpson</i>	<i>ACE</i>	<i>S evenness</i>	<i>Richness</i>
Aquatic / Terrestrial	.003	.001	.04	.81	.04
Roots / Shots	6.65 ⁻¹¹	1.37 ⁻¹¹	3.57 ⁻¹²	.02 ⁻³	3.25 ⁻¹²

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583 **Table 4.** Core microbiomes in roots, and soil samples, and percent identity with their closest match
 584 in the NCBI database.

Roots		Soil
<i>terrestrial</i>	<i>aquatic</i>	
Duganella sp. [100%]	Povalibacter sp. [95.71%]	Ktedonobacter sp. [94.39%]
Caulobacter rhizosphaerae [100%]	Methylovorus glucosotrophus [100%]	Psychrosinus sp. [98.13%]
Rhizobium cellulosilyticum [100%]	Novosphingobium sp. [100%]	Candidus Koribacter sp. [97.33%]
Novosphingobium sp. [100%]		Uncultured Acidobacteria 1
		Uncultured Acidobacteria 2
		Koribacter versatilis [98.76%]
		Uncultured Actinomycete 1
		Uncultured Actinomycete 2
		Uncultured Acidobacteria 3
		Ramlibacter ginsenosidimutans [100%]
		Acidovorax avenae [100%]
		Uncultured bacterium 1
		Psicinibacter sp. [100%]
		Bradyrhizobium japonicum [100%]
		Anaeromyxobacter sp.[98.7%]
		Anaeromyxobacter diazotrophicus [99.1%]
		Uncultured bacterium 2
		Geomonas subterranea [100%]
		Uncultured Deltaproteobacteria [99.8%]
		Uncultured Acidobacteracea [98.38%]
		Uncultured Actinobacteria [98.39%]

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598 **Table 5.** Ecological role of top ASVs associated with amphibious plants.

Bacterial taxa	Ecological role	Niche	% NCBI Identity	References
<i>Mucilaginibacter celer</i>	Improves growth and salt tolerance, some isolates also from freshwater ecosystems	All	100%	Kim et al, 2020
<i>Dawida soli</i>	Heterotrophic aerobic metabolism, endophytes of roots	Roots terrestrial and aquatic	99%	Manter et al, 2010
<i>Sphingomonas panacis</i>	Heavy metals resistance, rhizosphere resident	Aquatic roots, shoots and terrestrial roots	100%	Kim et al, 2019
<i>Acidisphaera rubrifaciens</i>	Acidic environments, aerobic, primary producer [bacteriochlorophyll-containing]	Aquatic roots, shoots and terrestrial shoots	97.86%	Hiraishi et al, 2000
<i>Chitinophaga pinesis</i>	Chitin degradation, fungal cell walls degradation	Roots	98%	Lu et al, 2023
<i>Sphingomona azotifigens</i>	Nitrogen fixing bacteria, growth promoter	Aquatic roots, shoots and terrestrial roots	99%	Videira et al, 2009
<i>Lichenicoccus roseus</i>	Antarctic lichen symbiont, xylan decomposer	Aquatic shoots	97.59%	Pankratov et al, 2020
<i>Uzinura diaspidicola</i>	Insects symbiont, nutrient acquisition	All	100%	Sabree et al, 2012
<i>Duganella vulcaria</i>	Endophytes roots and shoots, growth promoter	Aquatic roots, shoots and terrestrial roots	100%	Baldani et al, 2014
<i>Ideonelle azotifigens</i>	Rhizosphere, nitrogen fixer	Roots terrestrial and aquatic	100%	Noar & Buckley, 2009
<i>Rahnella variigena</i>	Endophyte, growth promoter	Roots terrestrial and aquatic	100%	Raj et al, 2023
<i>Filimonas aquilariae</i>	Plant roots resident, high CO ₂ dependent	All	99%	Lin et al, 2017
<i>Chitinophaga arvensicola</i>	Wetlands, plant detritivorous	Roots terrestrial and aquatic	98.38%	Pankratov et al, 2006
<i>Novosphingobium capsulatum</i>	Rhizosphere inhabitant, Sulfuric metabolism: alkaline sulfonate assimilation	Aquatic roots, shoots and aquatic shoots	99.46%	Kumar et al 2017
<i>Flavitalea</i> sp.	Root endophyte	Roots terrestrial and aquatic	98.65%	Fernandez-Gonzalez et al 2019
<i>Lichenicola cladoniae</i>	Cold adapted lichen symbiont (Antarctica)	All	100%	Noh et al, 2020
<i>Mucilaginibacter angelicae</i>	Rhizosphere, cellulose-degrading	Roots terrestrial and aquatic	100%	Kim et al 2012
<i>Mucilaginobacter jinjuensis</i>	Rotten wood xylan-degrading, latent in plant roots	Roots terrestrial and aquatic	100%	Khan et al, 2016

Niastella soli	Rhizosphere, denitrifier	Roots terrestrial and aquatic	99.9%	Akter et al, 2021
Caulobacter henricii	Fresh water and rhizosphere, associated with caulophages "Kronos"	Roots terrestrial and aquatic	100%	Berrios and Ely, 2019
Xanthomonas translucens	Plant pathogen	Roots terrestrial and aquatic	100%	Sapkota et al, 2020
Flavobacterium frigidarium	Sediments in Antarctica, detritivorous of plants and algae	Roots and less proportion shoots	100%	Humphry et al, 2001
Chryseobacterium aquaticum	Fresh water, see also Elizabethkingia	Roots terrestrial and aquatic	98%	Bernardet et al, 2006
Pantoea agglomerans	Associated with plant roots. It could be associated with human infections via wound infection with plant material.	Terrestrial shoots	100%	Cruz et al, 2007

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605 Vernal Pools and Grassland Reserve.

606

607

(A)



(B)



Figure 1. *Eryngium castrense* (Jeps.) is a California native plant, specialized to live under aquatic and desiccated environmental conditions. It grows in California vernal pools, which are temporary, Mediterranean-climate wetlands **(A)** “Isoetoide” aquatic morphology; **(B)** terrestrial morphology - “Spiny” weed.

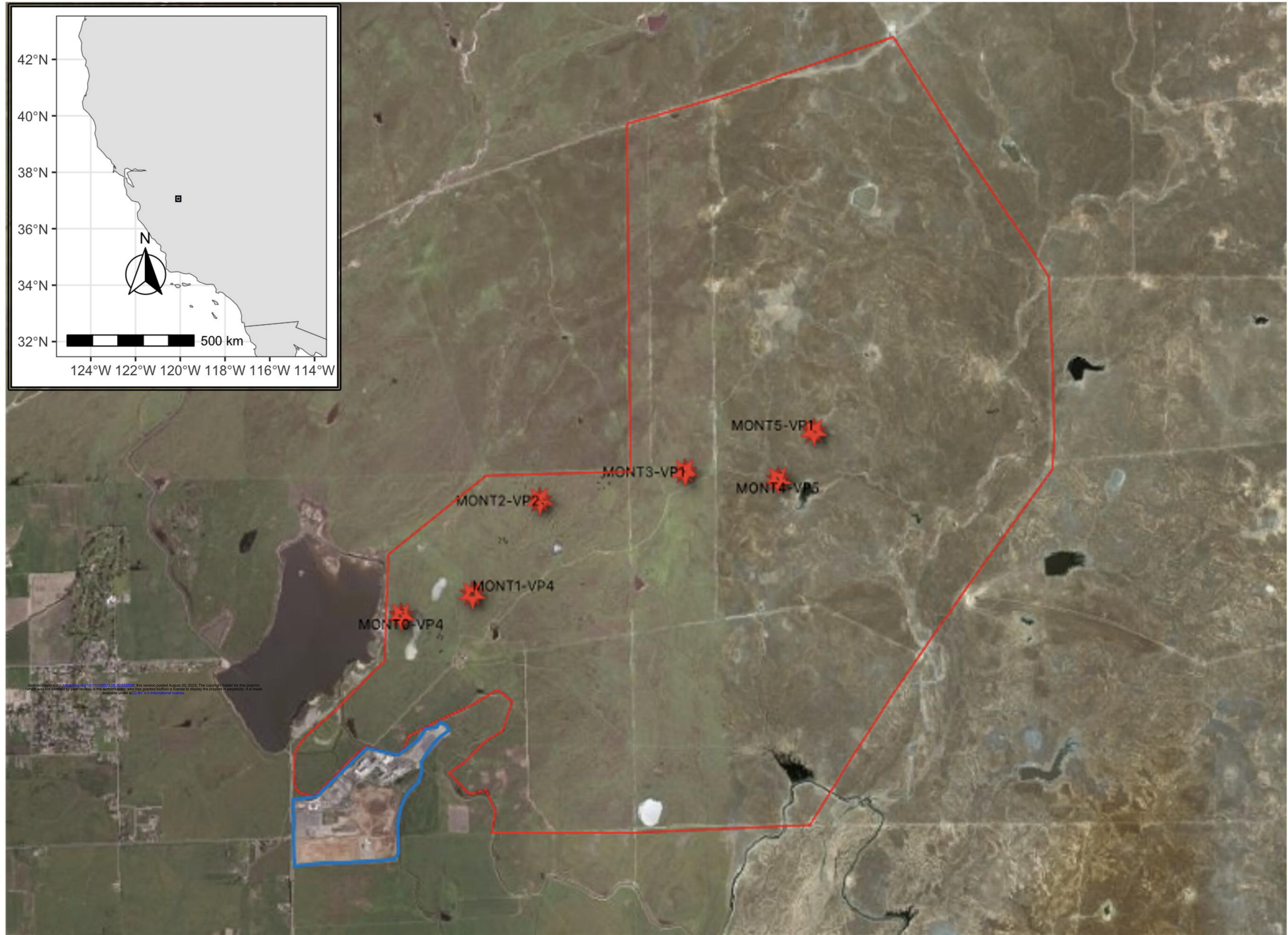


Figure 2. Map of the study site. UC-Merced Vernal Pools and Grassland Reserve (MVPGR) is delimited by the red polygon. The reserve is adjacent to the University of California campus, delimited with a blue polygon. Red marks indicate the location of vernal pools monitored for the studied microbes.

Dominant Phyla

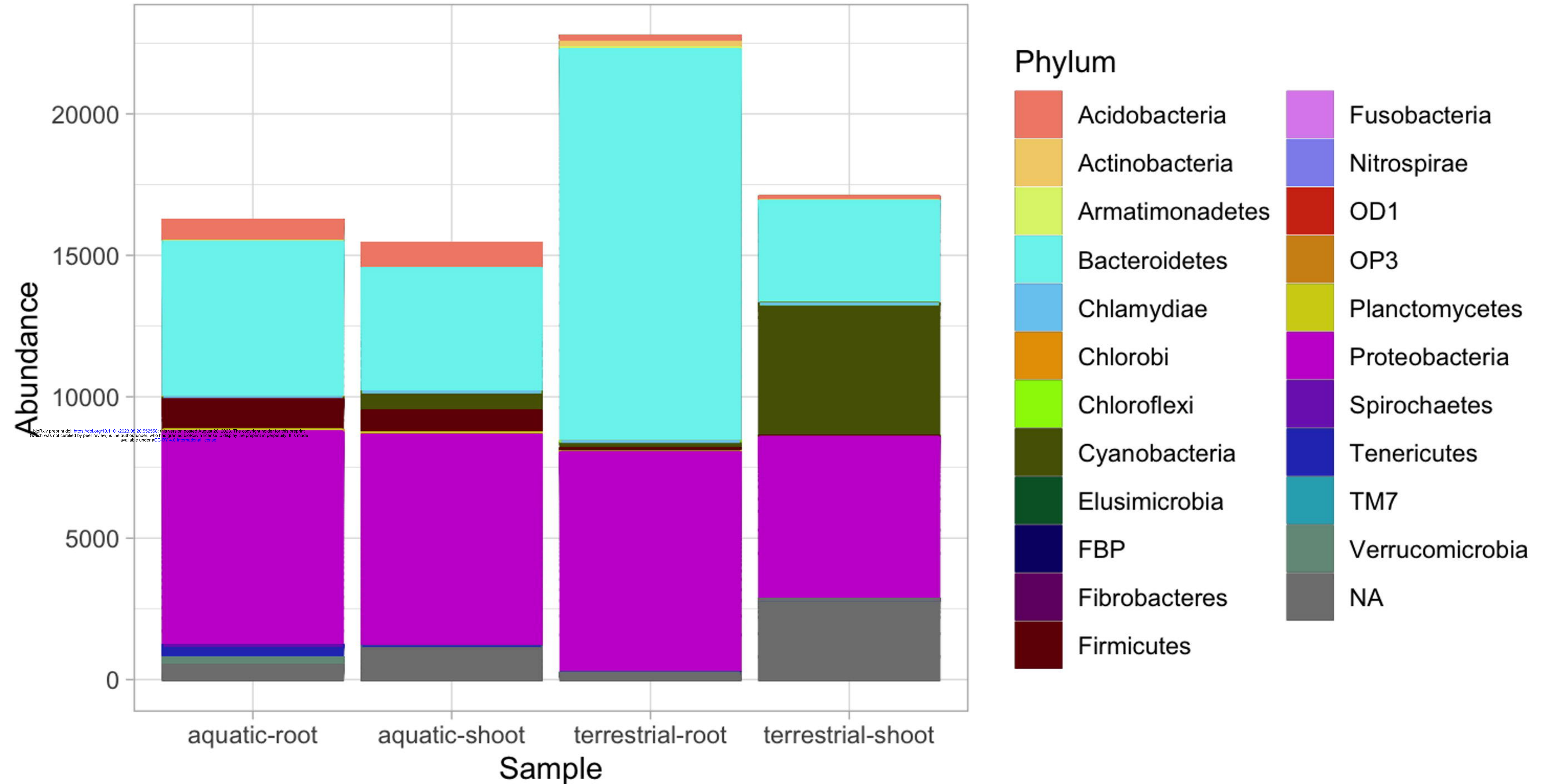


Figure 3. Dominant bacterial phyla in the amphibious plant *E. castrense*.

Bray-Curtis dissimilarity

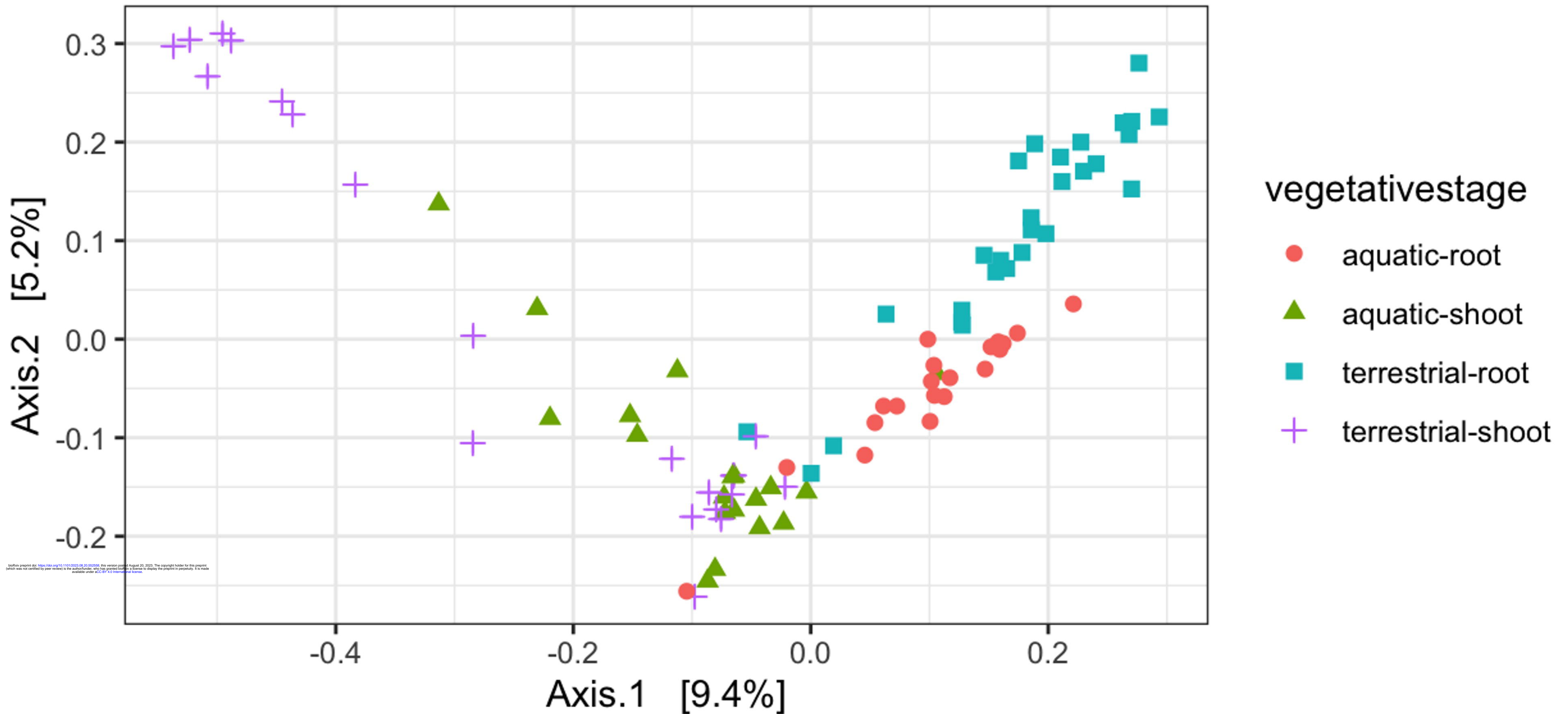


Figure 4. Principal Components Analysis (Bray-Curtis distances) for bacterial communities illustrates differences among plant compartments, as well as aquatic versus terrestrial phase. Samples are grouped and color shaded based on the combination of these factors.

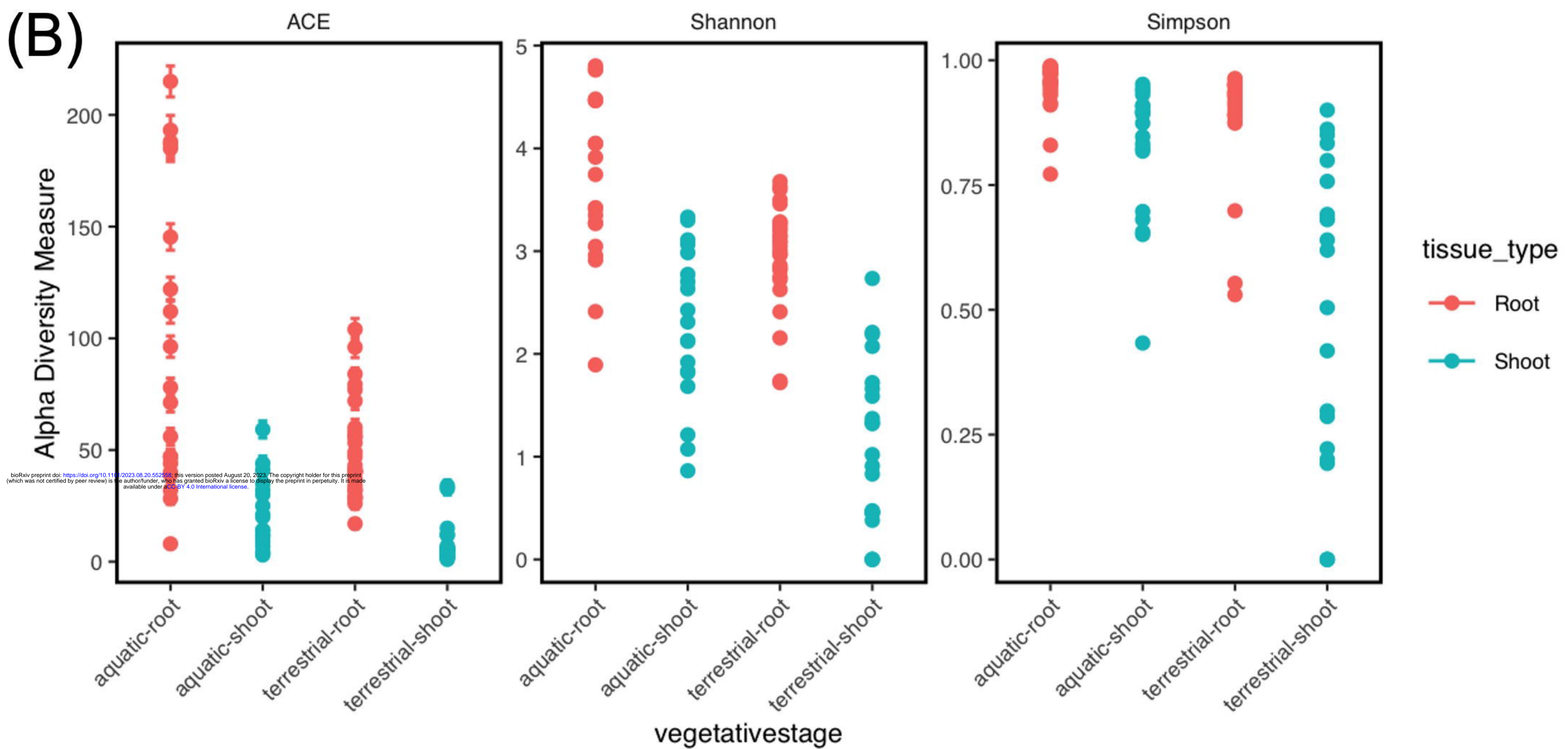
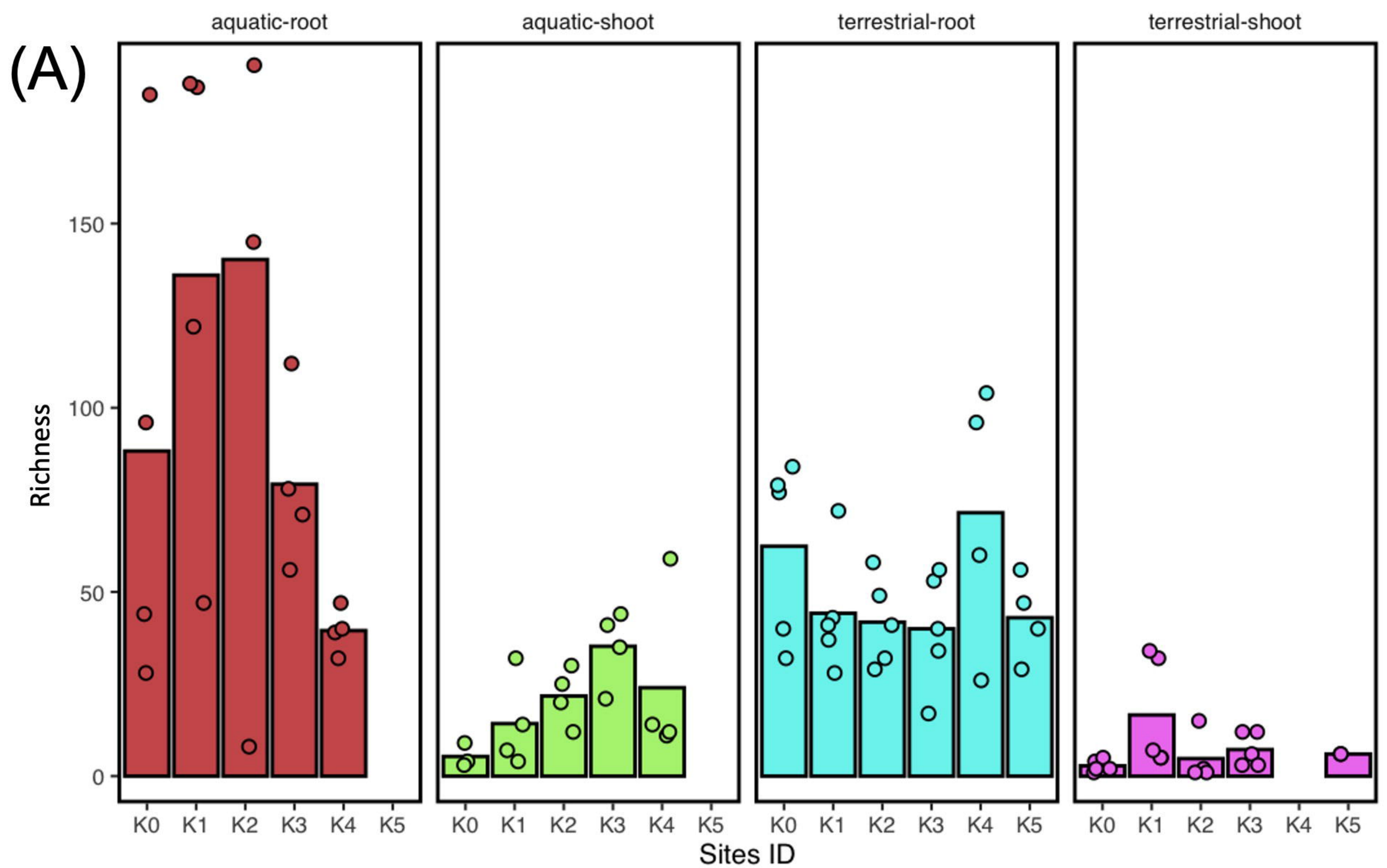


Figure 5. Alpha diversity in aquatic and terrestrial roots and shoots. **(A)** Differences in endophytic taxa diversity. Bars represent the total taxa abundance across sampling sites, given a specific tissue type and plant morphological stage. **(B)** Alpha diversity metrics of roots and shoots in both, aquatic and terrestrial. Dots represent individual samples.

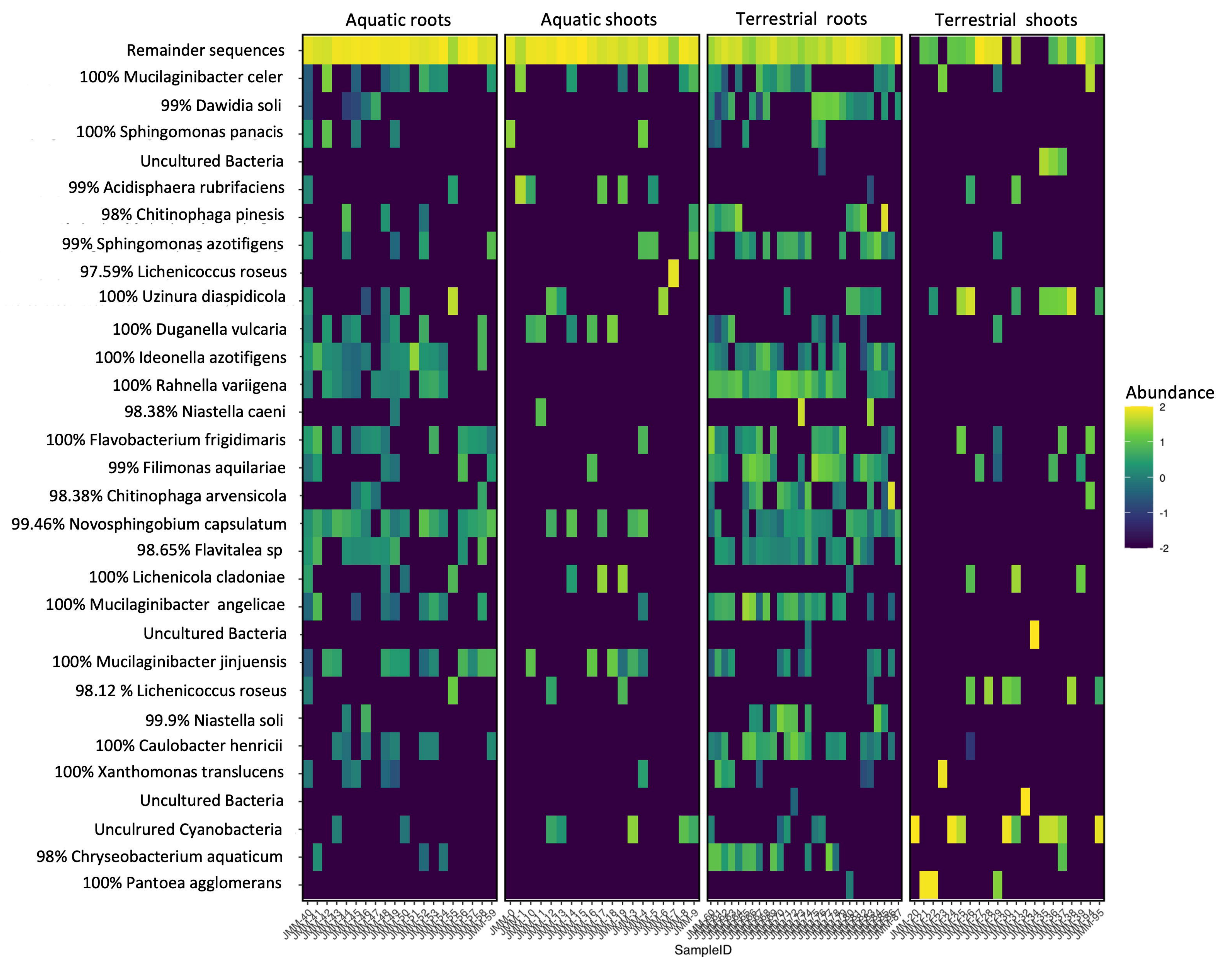
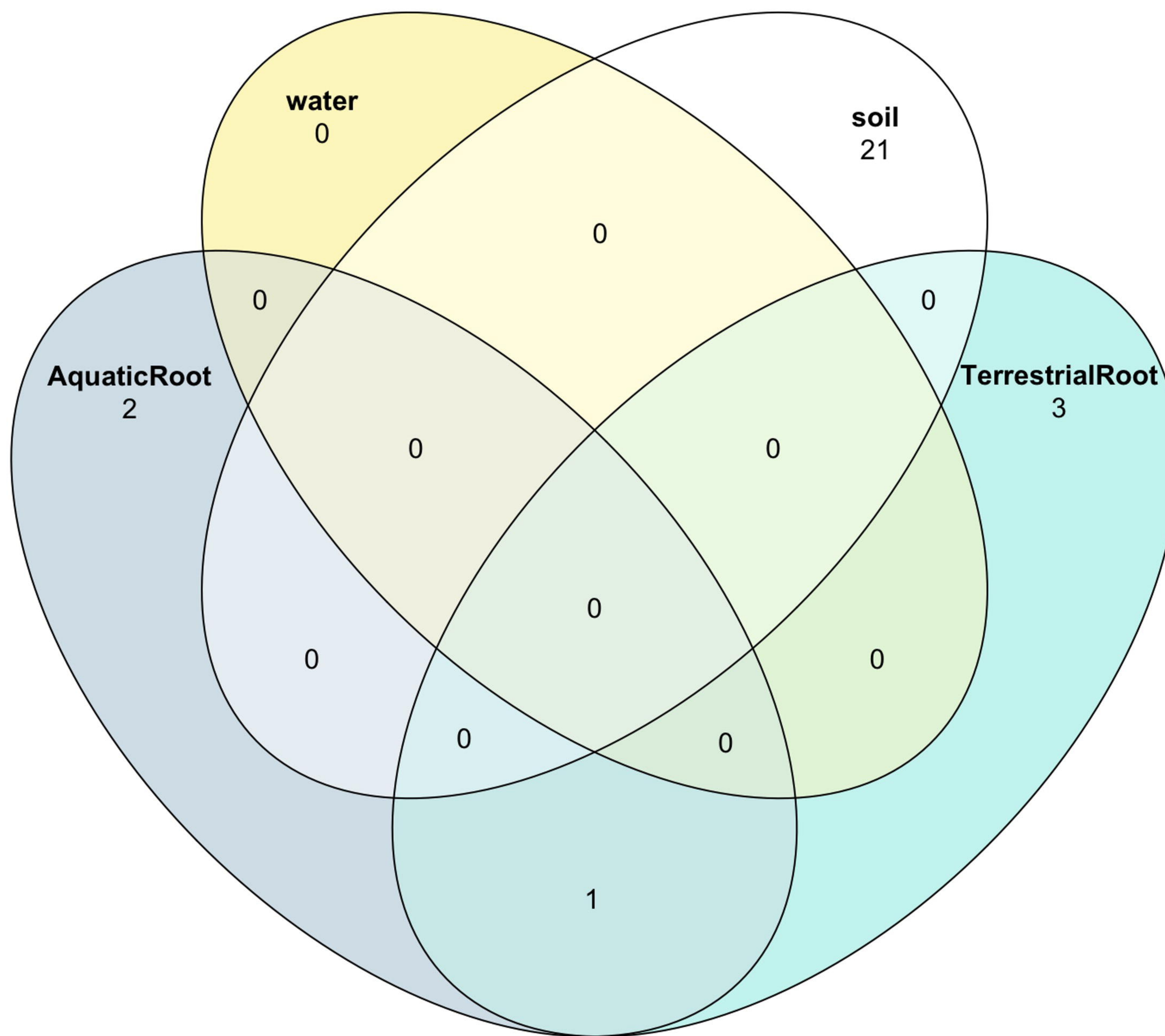


Figure 6. Heat map displaying the relative abundance of the 30 most abundant endophytic ASVs identified for the amphibious plant species *E. castrense*, organized by plant compartment. Percent identity to the closest match in NCBI is shown next to the assigned taxa name.



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Figure 7. Overlap in core microbiome by ecosystem compartment in vernal pools.

Supplementary Material

The microbiome of an amphibious plant shifts dramatically across the host life cycle.

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1 Supplementary Tables

Supplementary Table 1. Mantel test of spatial distance (geo position) and diversity metrics.

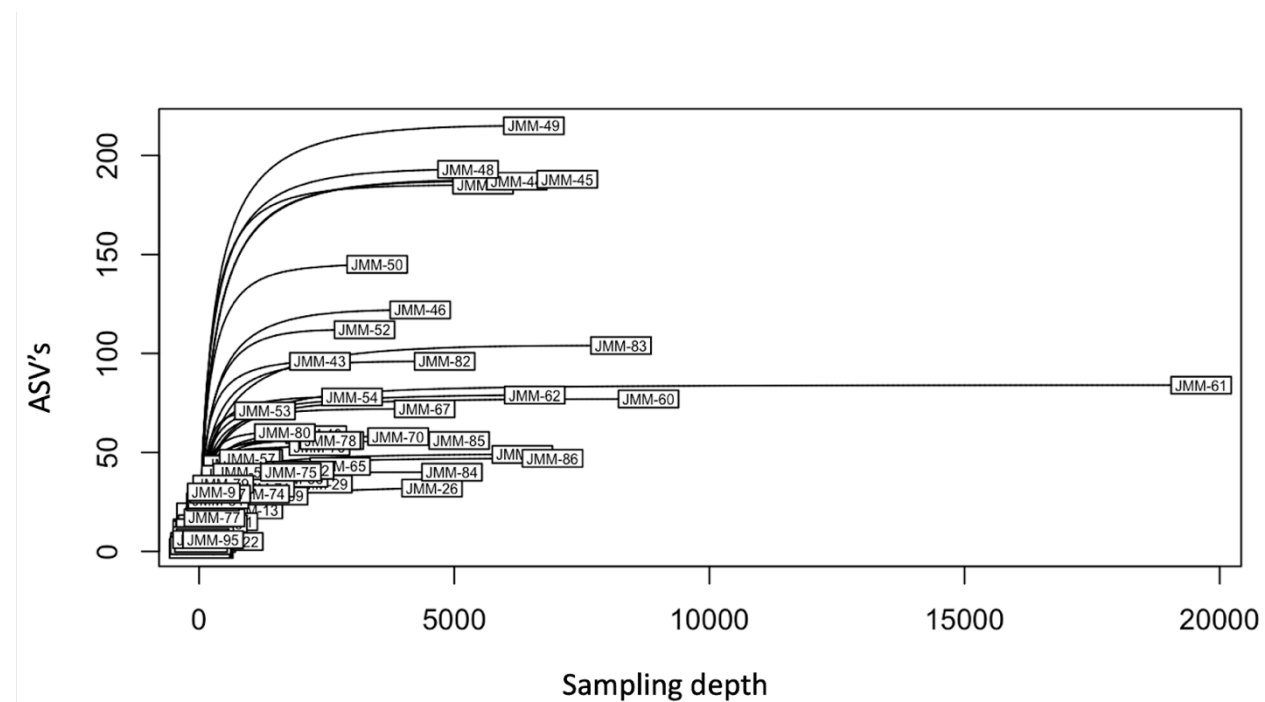
<i>Spatial distance Mantel</i>	<u>Observed</u>	<u>Shannon</u>	<u>Simpson</u>	<u>ACE</u>
Statistic r	-0.04	-0.04	0.06	-0.04
Significance	0.8	0.8	0.08	0.8

Supplementary Table 2. PERMANOVA-ADONIS by habitat type in vernal pool ecosystems, Root endosphere versus soil and water.

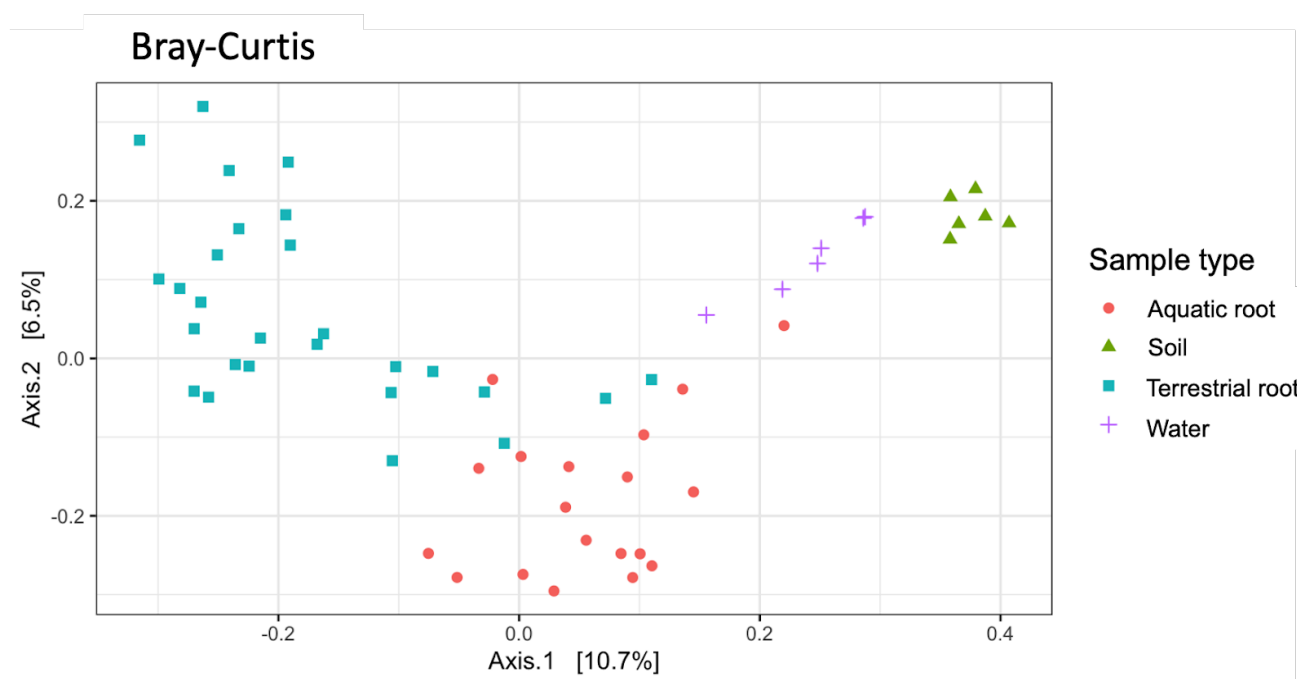
Permanova-ADONIS sample dissimilarity significance

<i>Dissimilarity method</i>	<i>Groups</i>	<i>SumsOfSq</i>	<i>MeanSqs</i>	<i>F.Model</i>	<i>R²</i>	<i>Pr(>F)</i>
Unw-Unifrac	3	4.05	1.35	5.77	0.24	0.001
Bray-Curtis	3	3.8	1.26	3.38	0.16	0.001

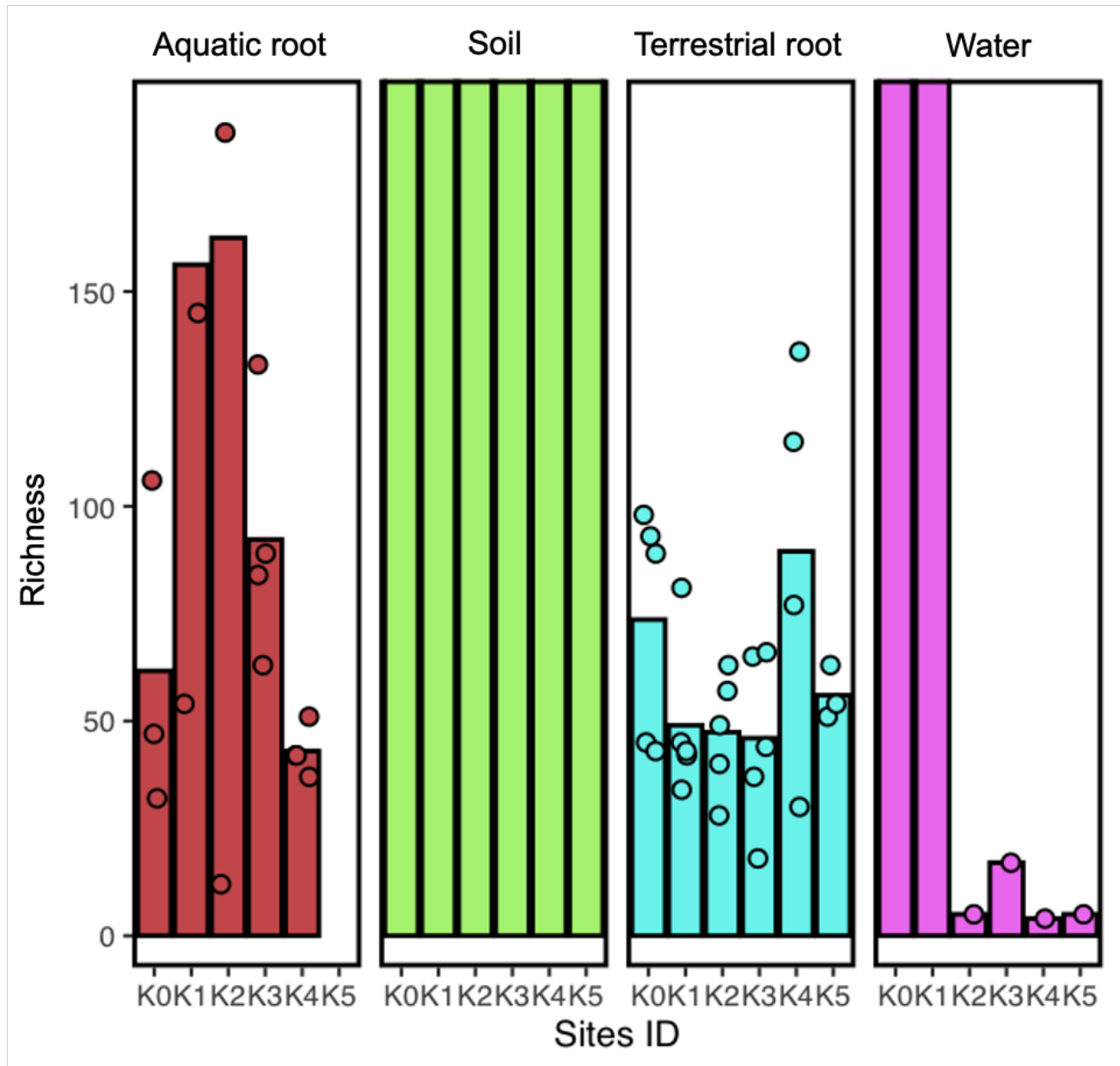
2 Supplementary Figures



Supplementary Figure 1. Sequence variants (ASV's) identified by the number of ASVs.



Supplementary Figure 2. PCA based on Bray-Curtis dissimilarity index between tissue samples, water and soil samples.



Supplementary Figure 3. Differences in diversity between ecosystem compartments, soil, water, and plant tissues.