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Microbiome of two predominant seagrass species of the Kenyan coast, *Enhalus acoroides* and *Thalassodendron ciliatum*

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Background. Metagenomics studies have reported on the complexity of microbiomes associated with seagrass and can provide critical insights into the sustainable use and conservation of seagrasses. Recent conservation activities in Kenya focused mainly on coral reefs and mangrove forests with little direct action taken to conserve seagrass meadows. Pollution, over-exploitation of marine resources and minimal efforts towards enforcement of conservation laws of marine environments, have caused degradation and defoliation of seagrass habitats. Little is known about the microbes associated with seagrass species in Kenya and this study aimed to characterize the genetic diversity of the microbiomes of two prominent seagrass species, *Enhalus acoroides* and *Thallasodendron ciliatum*, which are the most commonly occurring species.

Methods. Replicate microbiome samples were collected from leaves, roots, sediment and water columns associated with the two seagrass species from two sites on the Kenyan coast. The microbial communities of the samples were characterized and compared using 16S ribosomal RNA gene PCR and sequencing. Microbiome features including diversity and taxonomic composition were used to compare within and between sample types and sites.

Results. Leaf samples from both *E. acoroides* and *T. ciliatum* had significantly different microbial communities comparted to root and sediment samples, revealing a diversity gradient with lowest diversity in water samples and highest in sediment. There were no significant variation in seagrass microbial composition associated with leaf and rhizosphere microbiomes of either *E. acoroides* or *T. ciliatum*. However, we did see a difference between water samples associated with each seagrass species.

Discussion. This study of the microbiomes associated with the sediments, roots, leaves and surrounding water of *E. acoroides* and *T. ciliatum*, included a limited number of samples from a small geographic area, providing a valuable first assessment of the microbial diversity of seagrass beds on the Kenyan coast. We found no significant differences between the plant-associated bacterial communities of the two-seagrass species investigated. Significant differences however, were observed amongst leaf-, root-, sediment- and water-associated bacterial communities. This work will contribute to understanding the dynamic environment of seagrass beds and will contribute to helping conserving and re-establishing seagrass beds degraded by due to anthropogenic activities.

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Microbiomes of two predominant seagrass species of the Kenyan coast, *Enhalus acoroides* and *Thalassodendron ciliatum*

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Abstract

Background. Metagenomics studies have reported on the complexity of microbiomes associated with seagrass and can provide critical insights into the sustainable use and conservation of seagrasses. Recent conservation activities in Kenya focused mainly on coral reefs and mangrove forests with little direct action taken to conserve seagrass meadows. Pollution, over-exploitation of marine resources and minimal efforts towards enforcement of conservation laws of marine environments, have caused degradation and defoliation of seagrass habitats. Little is known about the microbes associated with seagrass species in Kenya and this study aimed to characterize the genetic diversity of the microbiomes of two prominent seagrass species, *Enhalus acoroides* and *Thallasodendron ciliatum*, which are the most commonly occurring species.

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41 geographic area, providing a valuable first assessment of the microbial diversity of seagrass beds
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47 activities.

48

49 Introduction

50 Around the world, coral reefs and seagrass beds protect shorelines and host highly productive
51 and diverse ecosystems. They provide hatcheries for and sustain marine species, including those
52 that we harvest for food. In Kenya, coral reefs cover approximately 600km^2 along the coast and
53 are often associated with seagrass beds, although the latter's total coverage has not been
54 determined. Seagrass communities are subject to frequent anthropogenic and natural
55 disturbances. Exposure to overexploitation, pollution and climate change can lead to alterations
56 in vegetation complexity, which in turn may affect associated fauna and microorganisms.
57 Documented recent seagrass losses along most of the Kenyan coast has been ascribed to
58 extensive grazing by sea urchins (*Tripneustes gratilla*) and this has caused habitat fragmentation
59 and defoliated beds (Mutisia, 2009). Limited transplantation projects were undertaken to counter
60 this trend and support natural recovery and Mutisia (2009) showed that defoliated seagrass beds
61 could recover fully in density, diversity and community structure of its meiofauna and
62 specifically of harpacticoid copepods.

63 Recent studies have shown that when disruptions occur in an ecosystem, associated changes in
64 the microbial communities or microbiomes, can be at least equally or more significant than that
65 of the macrofauna and -flora. Documenting and understanding such shifts in the composition and
66 abundance of individual species within a microbiome, can provide insights and likely identify
67 key microorganisms, of which the presence and abundance act as indicators of the environment's
68 health (Orth *et al*., 2006).

69 Following this line of reasoning, Uku *et al*. (2007) assessed the presence and abundance of
70 prokaryotic epiphytes on leaves of three seagrass species in Kenya, *Thalassodendron ciliatum*,
71 *Thalassia hemprichii* and *Cymodocea rotundata* and how they varied between sites containing
72 differing levels of nutrients that were associated with human activities, in the water. They used
73 denaturing gradient gel electrophoresis (DGGE) and PCR amplified 16S rRNA gene fragments
74 and found higher epiphytic coverage of seagrass leaves was associated with water with more
75 nutrients, while the microbial diversity was linked to seagrass species rather than the study sites.

76 Metagenomics studies have reported on the complexity of microbiomes associated with seagrass
77 (Cúcio, *et al*., 2016) and how they can be used as pointers and drivers of the biogeochemical
78 environment within biofilms, such as those associated with many marine organisms (Agler, *et*
79 *al.,* 2016). Knowledge of the microbial composition of these communities and how they fluctuate
80 with changes in the environment can provide critical insights into the sustainable use and
81 conservation of seagrasses.

On the Kenyan coast, pollution, over-exploitation of marine resources and minimal efforts towards enforcement of conservation laws of marine environments, have caused degradation and defoliation of seagrass habitats (Tuda & Omar, 2012). Recent conservation activities in Kenya focused mainly on coral reefs and mangrove forests with little direct action taken to conserve seagrass meadows, despite the fact that they are valued 3 times higher than coral reefs and 10 times more than tropical forests (Björk, *et al.,* 2008).

This study reports on the microbiomes of two prominent seagrass species on the Kenyan coast. Since little is known about the microbes associated with seagrass species in Kenya, this study aimed to characterize the genetic diversity of the microbiomes of two prominent seagrass species, *Enhalus acoroides* which is eaten as a snack in Lamu county and *T. ciliatum*, which is the most commonly occurring species.

Materials & Methods

Seagrass sampling and DNA isolation

Baobab beach (-3.629383, 39.872271) is a public beach and Kuruwitu Marine Sanctuary (-3.808319, 39.831288) is a protected reef in Kilifi county, Kenya with extensive seagrass beds of *T. ciliatum* at Baobab beach and *E. acoroides* at Kuruwitu Marine Sanctuary. Two replicate microbiome samples were collected from leaves, roots, sediment (rhizobiome) and water columns of each species. DNA extraction was done with a Powersediment DNA extraction kit (MoBio Laboratories) for sediment, leaf and root microbiomes as recommended by Ettinger, *et al.* (2014) and a Powerwater DNA extraction kit (MoBio Laboratories) was used for microbes collected onto 0.22μm filter membranes from water samples.

16S rRNA gene amplicon sequencing

The Ion 16S Metagenomics kit (ThermoFisher Scientific) with primer set V2-4-8 was used to amplify the 16S variable regions for all samples according to the kit instructions. PCR products were purified with an AMPure magnetic bead purification system (Beckman Coulter) and quantified with the QuBit dsDNA HS Assay kit and Fluorometer (ThermoFisher Scientific). Libraries were synthesized and barcode adaptors added with the Ion Plus™ Fragment Library Kit (ThermoFisher Scientific). Each barcoded library was assessed to confirm the ligation of adapters (Agilent Bioanalyzer, Agilent) and concentration of libraries (Ion Library TaqMan® Quantitation Kit, ThermoFisher Scientific). Barcoded libraries were pooled in equimolar amounts and one positive control (Microbial Mock Community A) added to one batch. Templating of the pooled library was done using the Ion PGM™ Hi-Q™ OT2 Kit on the OneTouch2™ system as per the manufacturer's recommendation (ThermoFisher Scientific). Sequencing of 400bp fragments was performed on a 318 v2 chip using an Ion PGM and Hi-Q™ Sequencing Kit (ThermoFisher Scientific). Base calling and run demultiplexing was performed with Torrent_Suite software version 5.0.4 with default parameters for the General Sequencing application.

Sequence processing and taxonomic assignment

Individual sequence reads were filtered to remove low quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were automatically trimmed. All PGM quality-

124 approved, trimmed and filtered data were exported as sff files and further visualised and trimmed
125 on quality in CLC Genomics Workbench version 9.5.3, exported as fasta files and processed
126 using QIIME version 1.9.1 (Caporaso *et al*., 2010). Sequences with a length between 200 and
127 250 bp with mean sequence quality score of > 25 were retained. Sequences with homopolymers
128 of more than 6 bp or with mismatched primers, were omitted. 16S rRNA Operational Taxonomic
129 Units (OTUs) were defined at ≥ 97% sequence homology using the open reference picking
130 pipeline and taxonomy assignment workflow of QIIME and the Greengenes database (v.13_8)
131 (DeSantis *et al*., 2006). Data-mining and statistical analysis was done in Calypso version 8.54
132 (http://bioinfo.qimr.edu.au/calypso/) including visualization of the taxonomic information
133 (Zakrzewski *et al*., 2017). Core microbiomes were analysed using MetaCoMET with an absolute
134 read count threshold > 100 and using the membership method for calculating the core OTUs
135 (https://probes.pw.usda.gov/MetaCoMET/index.php).
136

137 **Results**

138 **Intra-sample variations between seagrass species**

139 The 16S rRNA amplicon sequencing generated a total of 1,404,280 high-quality reads from 16
140 samples with a median read count per sample of 78,207 (range: 35,783 – 155,095). High-quality
141 reads were clustered using > 97% sequence identity into 2,312 microbial OTUs. OTUs with less
142 than one percent relative abundance across all samples were discarded, resulting in 2,112 OTUs.
143 OTU counts were normalized using total sum normalization (TSS), which divides feature read
144 counts by the total number of reads in each sample.

145 To estimate the representativeness of our data set and to assess if there were enough sequences to
146 capture the intra-sample community richness, we performed a rarefaction analysis for the
147 identified OTUs (Fig. 1A). All samples attained sufficient sequencing depth to capture the
148 microbial diversity since all the graphs tapered off towards a horizontal line. Alpha diversity
149 metrics of species richness and evenness (Chao1, $P = 0.236$, ANOVA, Shannon, $P = 0.000642$
150 ANOVA and Simpson, $P = 0.000231$, ANOVA) were determined for each sample (Fig. 1 B, C
151 and D) revealing a diversity gradient with lowest diversity in water samples and highest in
152 sediment.

153 **Inter-sample variation between seagrass species**

154 Principal coordinates analysis (PCoA) based on Bray-Curtis distance metric of OTUs, revealed
155 that distinct microbial communities were associated with either *E. acoroides* or *T. ciliatum*
156 leaves or rhizosphere (root and sediment) samples (Fig. 2). PERMANOVA tests performed on
157 three different beta diversity metrics - Bray Curtis dissimilarity, unweighted unifrac and
158 weighted unifrac - confirmed that these communities were significantly different from each other
159 with $p = 0.001$ (Table 1). Leaf samples from both *E. acoroides* and *T. ciliatum* were significantly
160 different from root and sediment samples, which clustered together and distinct from water,
161 which clustered separately. However, there were no significant variation in seagrass microbial
162 composition associated leaf and rhizosphere microbiomes of either *E. acoroides* or *T. ciliatum*.
163 However, we did see a difference between water samples associated with each seagrass species.

164 **Major patterns in microbiome compositions of leaves and rhizobiomes**

165 Results from the diversity metrics presented above showed that there were distinct microbial
166 communities associated with leaves, rhizobiomes and water. We further investigated this by
167 determining in more detail the taxonomic composition of the phylum of microbes associated

168 with each sample type. Figure 3 and Table S1 summarize the mean relative abundance of
169 different phyla of bacteria for leaf and rhizosphere associated bacteria. The most abundant
170 bacteria associated with leaf samples were from the phyla *Proteobacteria* (61.8%),
171 *Cyanobacteria* (25.9%) and *Bacteroidetes* (8.1%). In comparison, root microbiomes contained
172 predominately *Proteobacteria* (73.9%), *Cyanobacteria* (8.7%), *Actinobacteria* (3.6%),
173 *Acidobacteria* (1.0%) and *Fibrobacteres* (1.4%). Sediment samples contained a very similar
174 bacterial composition to that of roots - *Proteobacteria* (64.0%), *Cyanobacteria* (8.0%),
175 *Actinobacteria* (3.6%), *Acidobacteria* (2.9%) and *Fibrobacteres* (1.9%) - but with additional
176 *Acidobacteria* (2.9%), *Planctomycetes* (2.3%), *Gemmatimonadetes* (1.6%) and *Chloroflexi*
177 (1.0%) bacteria. Water samples contained predominately *Proteobacteria* (59.2%), *Bacteroidetes*
178 (29.7%), *Cyanobacteria* (7.5%), *Fusobacteria* (1.2%) and *Actinobacteria* (1.2%.)

179 The most abundant classes of epiphytes associated with leaf samples were *Alphaproteobacteria*
180 (49.3%), *Chloroplast* (22%), *Gammaproteobacteria* (9.9%), *Saprospirae* (5.5%) and
181 *Flavobacteriia* (2.8%) (Fig. 4 and Table S2). Rhizobiome samples had mostly
182 *Gammaproteobacteria* (39.4 - 28.9%), *Alphaproteobacteria* (20.9 – 17.7%), *Deltaproteobacteria*
183 (10.4 – 16.8%), *Chloroplast* (10.4 – 16.8%) and *Acidimicrobiia* (6.7 – 4.9%) respectively. Water
184 microbiomes, in contrast, consisted predominately of *Alphaproteobacteria* (34.8%),
185 *Flavobacteriia* (28.5%), *Gammaproteobacteria* (21.1%) and *Chloroplast* (5.1%).

186 **Variation in water microbial communities between seagrass species**

187 One-way ANOVA multiple comparison tests indicated differences in relative abundance of
188 microbial communities in seawater samples associated with *E. acoroides* or *T. ciliatum* (Table
189 S1). Water samples from *E. acoroides* had significantly more *Cyanobacteria* (12.2%) and
190 *Actinobacteria* (1.8%) compared to water from *T. ciliatum*, which in comparison had more
191 *Bacteroidetes* (40.3%).

192 **Leaf and root associated core microbiome of seagrass species**

193 The bacterial communities of root and leaf samples from the two-seagrass species were
194 compared in order to reveal a core microbiome. The top six most abundant core OTUs
195 (abundance between 66 and 1.8 % of the core microbiome) were classified as members of six
196 different phyla, *Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria, Acidobacteria*
197 *and Planctomycetes*, summarized in Fig. 5 A and B.

198

199 **Discussion**

200

201 Seagrass communities are essential to their environments; they fix organic carbon and sequester
202 atmospheric CO_2, cycle nutrients, stabilize submerged sediments along shores and provide
203 habitats for many organisms (Orth *et al*., 2006). These communities are often perturbed by
204 human and natural disturbances that alter vegetation complexity and its associated fauna
205 (Mutisia, 2009). Our current knowledge of how microbiomes facilitate seagrass microbial
206 community recovery following disturbance or restoration, is limited. In south Florida, USA,
207 Bourque *at al*. (2015) showed that community structure and diversity varied with sediment
208 depth, restoration treatments and over time. Their results indicated that microbial communities in
209 seagrass meadows changed in response to physical disturbance of the rhizosphere, and that

210 common restoration techniques led to the formation of distinct microbial communities during the
211 first year of recovery.

212 Gribben *et al.* (2017) found that microbial communities associated with *Zostera capricorni* in
213 Australia changed due to the presence of the invasive alga *Caulerpa taxifolia* and that healthy *Z.*
214 *capricorni* sediment microbial communities seemed to help defend the seagrass from invasion by
215 the alga. Microbial communities associated with seagrass species therefore appear to indirectly
216 control the success of establishing new seagrass beds.

217 Several studies demonstrated close relationships between individual seagrass species and their
218 microbial communities. Most of these focused on species from North America, Europe, China
219 and the Red Sea, including *Z. marina*, *Z. japonica*, *Cymodocea nodosa* and *T. hemprichii*
220 (Crump *et al*. 2018; Cucio *et al*. 2016; Yu-Feng Jiang *et al*. 2015; Ettinger *et al*. 2017; Rotini *et*
221 *al*. 2016). However, little is known about these associations between microbiomes and
222 seagrasses species occurring in Kenya. The only documented study investigated the prokaryotic
223 epiphytes on leaves of three seagrass species, *T. ciliatum*, *T. hemprichii* and *C. rotundata* using
224 denaturing gradient gel electrophoresis (DGGE) and clone libraries of PCR- amplified 16S
225 rRNA gene fragments (Uku *et al*., 2007).

226 In this study we report on the composition of microbial communities associated with *T. ciliatum*
227 and *E. acoroides*, two prominent seagrass species found along the Kenyan coast. When species
228 diversity was considered at a local scale (alpha diversity), it was highest in samples from
229 sediment, followed by roots, leaves and lowest in water. This was similar to what was generally
230 observed in terrestrial systems (Edwards *et al.*, 2015) and for other seagrass species such as *Z.*
231 *marina* (Ettinger, *et al.*, 2017).

232 The beta-diversity of *T. ciliatum* and *E. acoroides* microbiomes showed many differences
233 between the composition of leaf and rhizosphere communities, consistent with findings from
234 other seagrass microbiome studies (Crump *et al*., 2018; Cucio *et al*., 2016; Yu-Feng Jiang *et al*.,
235 2015; Ettinger *et al*., 2017; Rotini *et al*. 2016). Tight clustering of rhizosphere replicates was
236 consistent for both species in the PCoA based on Bray-Curtis distance metrics (Fig. 2) and
237 distinct from that of the water and leaf microbiomes of either species. We observed no
238 discernable differences in the microbiomes of the different seagrass species collected from the
239 two sites, except for water samples, which clustered separately. This corresponded to the results
240 of Cucio *et a*l. (2016) and Crump *et al*. (2018) which showed that although rhizosphere
241 microbiome compositions were significantly different from those of the phylospheres in these
242 seagrass species, there was no significant differences between corresponding microbiomes of
243 different seagrass species. This was contradictory to the findings of Ettinger *et al*. (2017) where
244 microbiomes of the rhizospheres differed significantly between samples and in sediments from
245 different sites.

246 Similar to the report of Crump *et al*. (2018), we found significant differences between leaf and
247 water microbiomes with water-associated microbial samples dominated by *Bacteroidetes*,
248 *Alphaproteobacteria and Gammaproteobacteria.* In addition, we found *Flavobacteria*
249 predominately associated with *T. ciliatum*. This was also previously noted by Uku *et al*. (2007)
250 to be associated with *T. ciliatum* and *T. hemprichii* in nutrient-poor sites. Our finding thus
251 support those of Crump *et al*. (2018) that exchanges occur between the submerged seagrass leaf
252 microbiomes and its associated water column, and that leaf microbiomes may act as a source of
253 organisms for local bacterioplankton.

254 The top six most abundant core OTUs, which also belonged to six different phyla were
255 *Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria, Acidobacteria and*
256 *Planctomycetes*. This corresponded with the results of Cúcio *et al*. (2016) and Mejia *et al*. (2016)
257 which indicated that the same phyla formed part of the core microbiomes of the rhizospheres of
258 *Z. marina, Z. noltii* and *Halophila stipulacea.* Since the fertility of sandy beach sediments are
259 generally poor (Perry and Dennison, 1999), seagrasses rely on nutrients fixed by bacteria
260 associated with them. Some of these, which were observed previously and also in this study,
261 included the free-living aerobic nitrogen fixing genus *Cyanobacteria* (Farnelid, 2013) and
262 anaerobic nitrogen fixing phyla C*hloroflexi* and *Chlorobi* (Foster and Mullan, 2008). These were
263 observed in relatively higher proportions associated with *E. acoroides* compared to *T. ciliatum*
264 except for *Chlorobi*, which was more abundantly associated with *T. ciliatum*.

265 **Conclusions**

266 This study of the microbiomes associated in the sediments, roots, leaves and surrounding water
267 of *E. acoroides* and *T. ciliatum*, included a limited number of samples from a small geographic
268 area, providing a valuable first assessment of the microbial diversity of seagrass beds on the
269 Kenyan coast. We found no significant differences between the plant-associated bacterial
270 communities of the two-seagrass species investigated. Significant differences however, were
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274 activities.

275
276

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Table 1 (on next page)

Permanova results. Comparing microbial community composition between different sample types (leaf, root, sediment)

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1

Diversity metric	Pseudo-F	R2	P (perm)
Bray Curtis	3.8981	0.77329	0.001
Unweighted unifrac	2.3442	0.67226	0.006
Weighted unifrac	4.3671	0.79258	0.001

2

Figure 1 (on next page)

Community richness and alpha diversity

(A) Community richness is estimated by rarefaction analysis to account for differences in sample sizes. Metrics used for measuring microbial alpha diversity, included (B) Chao 1, (C) Simpson index and (D) Shannon index.

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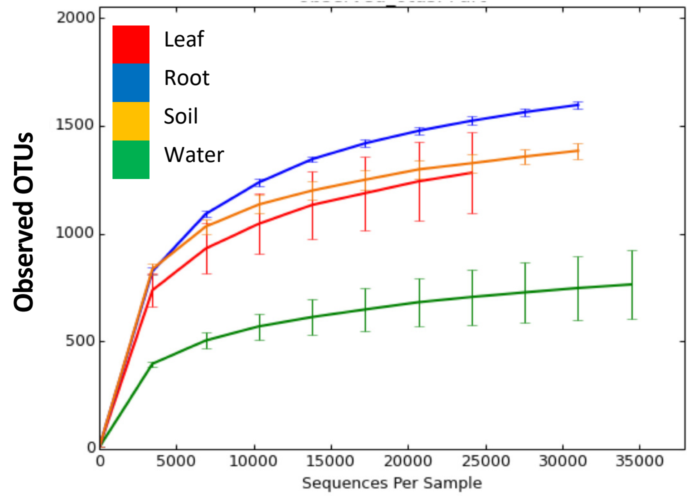

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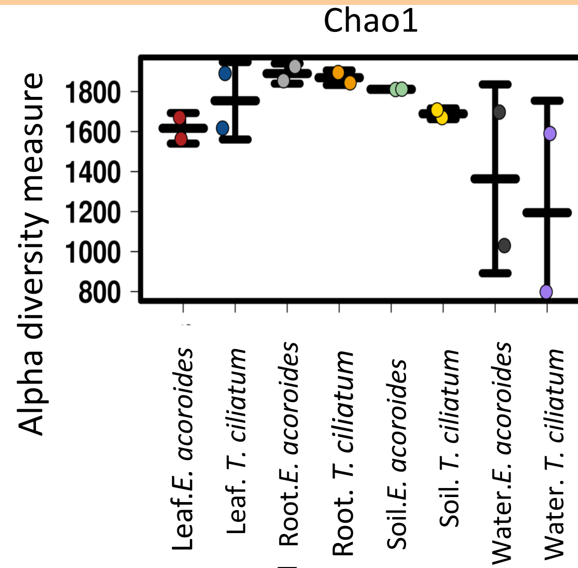

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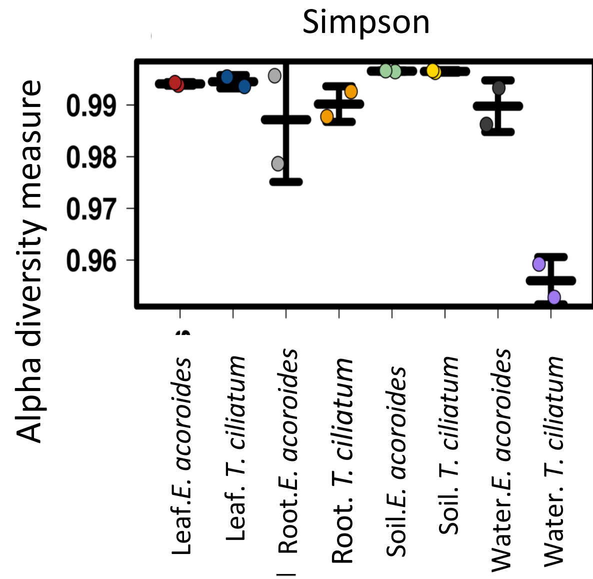

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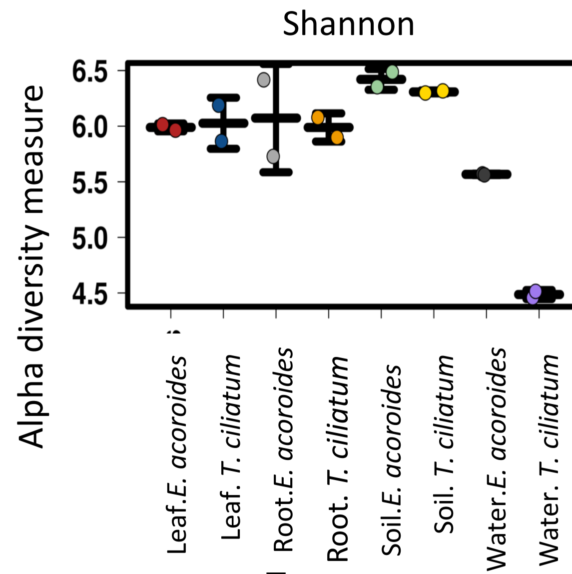

D

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Figure 2(on next page)

Principal coordinates analysis (PCoA) of the seagrass microbiome samples

PCoA was based on analyses of both V2-4-8 hypervariable regions of the prokaryotic 16S rRNA gene and was coloured based on sample site and species of seagrass.

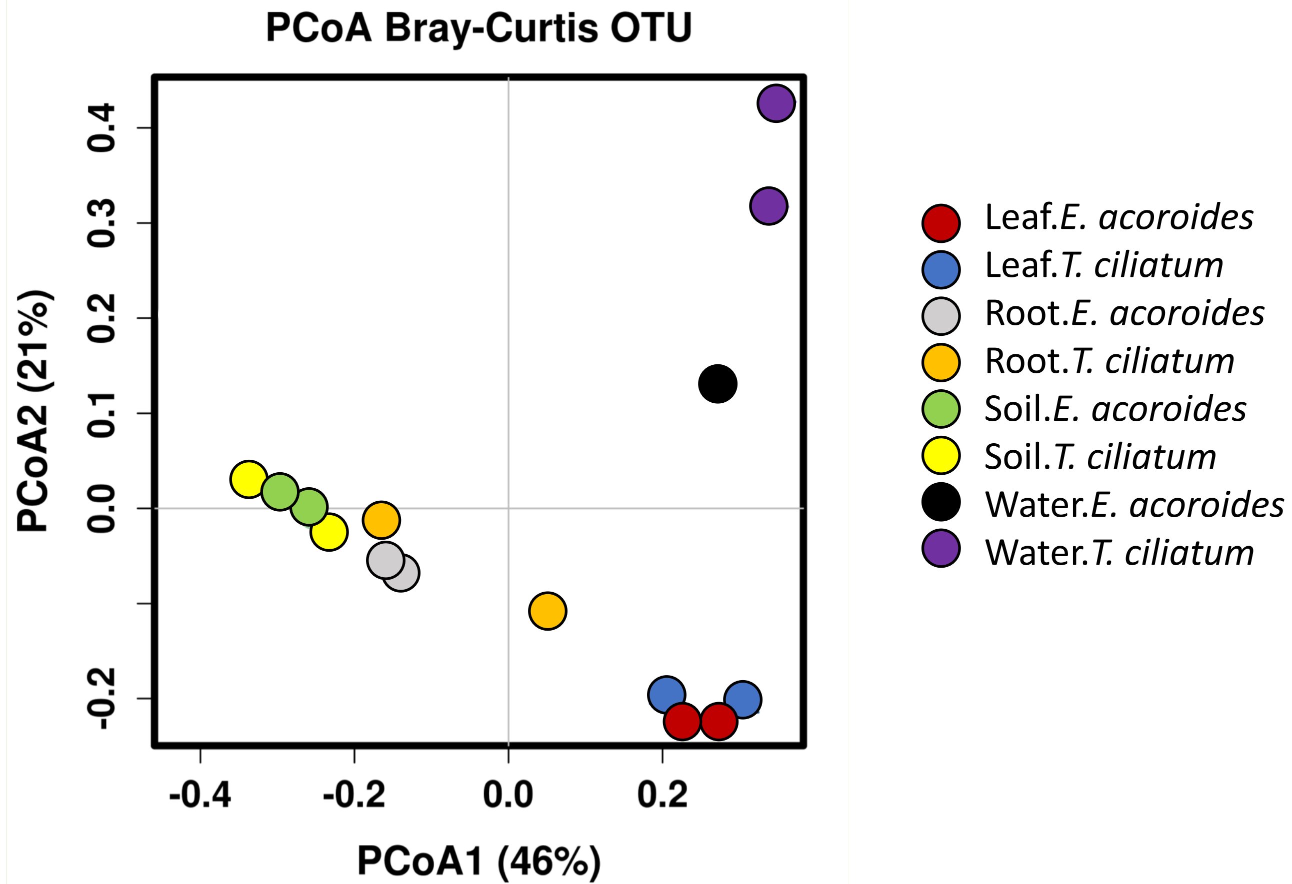

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Figure 3 (on next page)

Relative abundance of the dominant bacterial groups based on phylum

Box plots showing the taxonomic diversity of the dominant bacterial groups based on phylum level associated with leaf, root, sediment and water samples from *T. ciliatum* and *E. acoroides.* Bolded names indicate the most abundant phyla.

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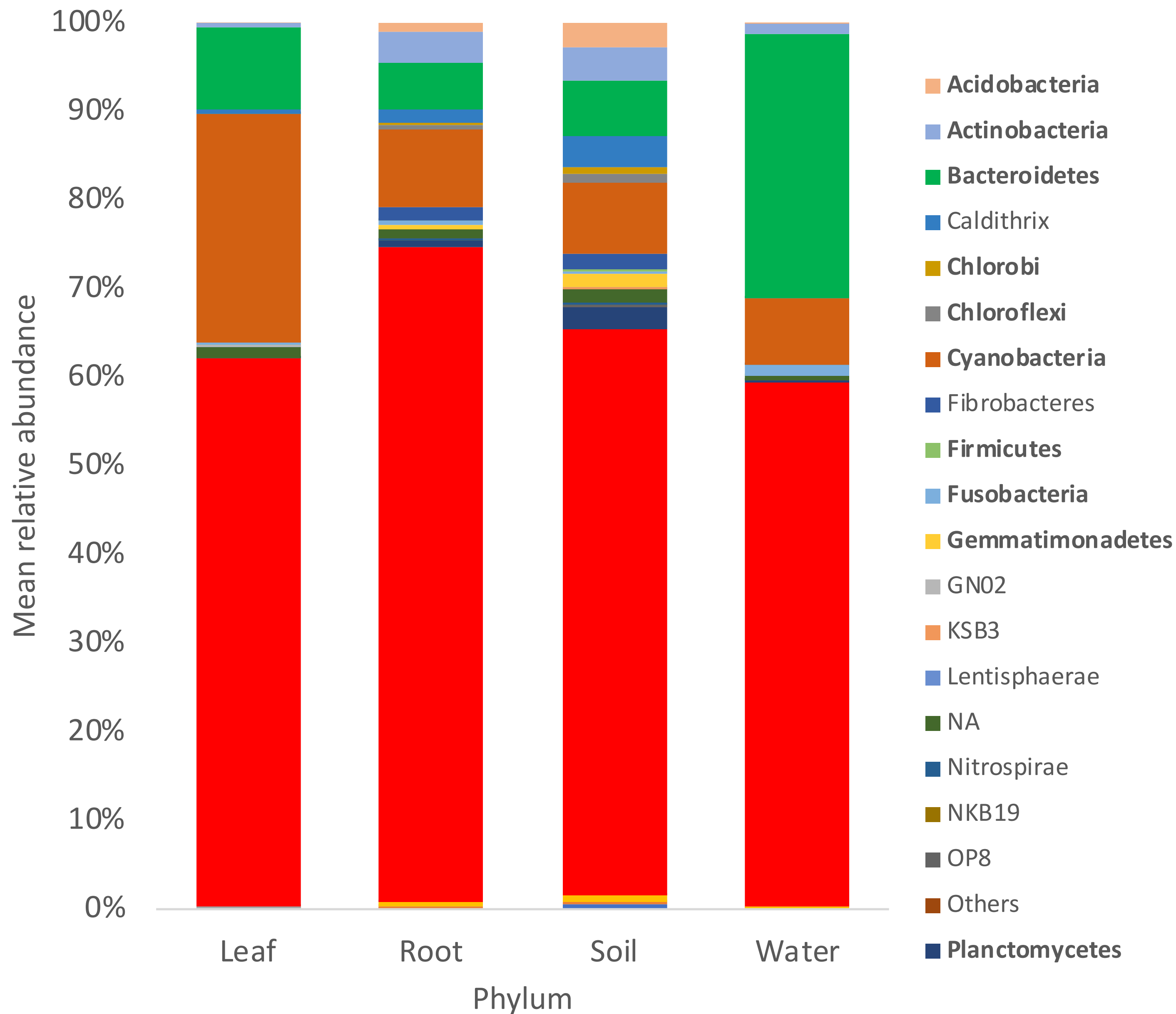

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Figure 4

(on next page)

Relative abundance of the dominant bacterial groups based on class

Box plots showing the taxonomic diversity of the dominant bacterial groups based on class level associated with leaf, root, sediment and water samples from *T. ciliatum* and *E. acoroides.* Bolded names indicate the most abundant class.

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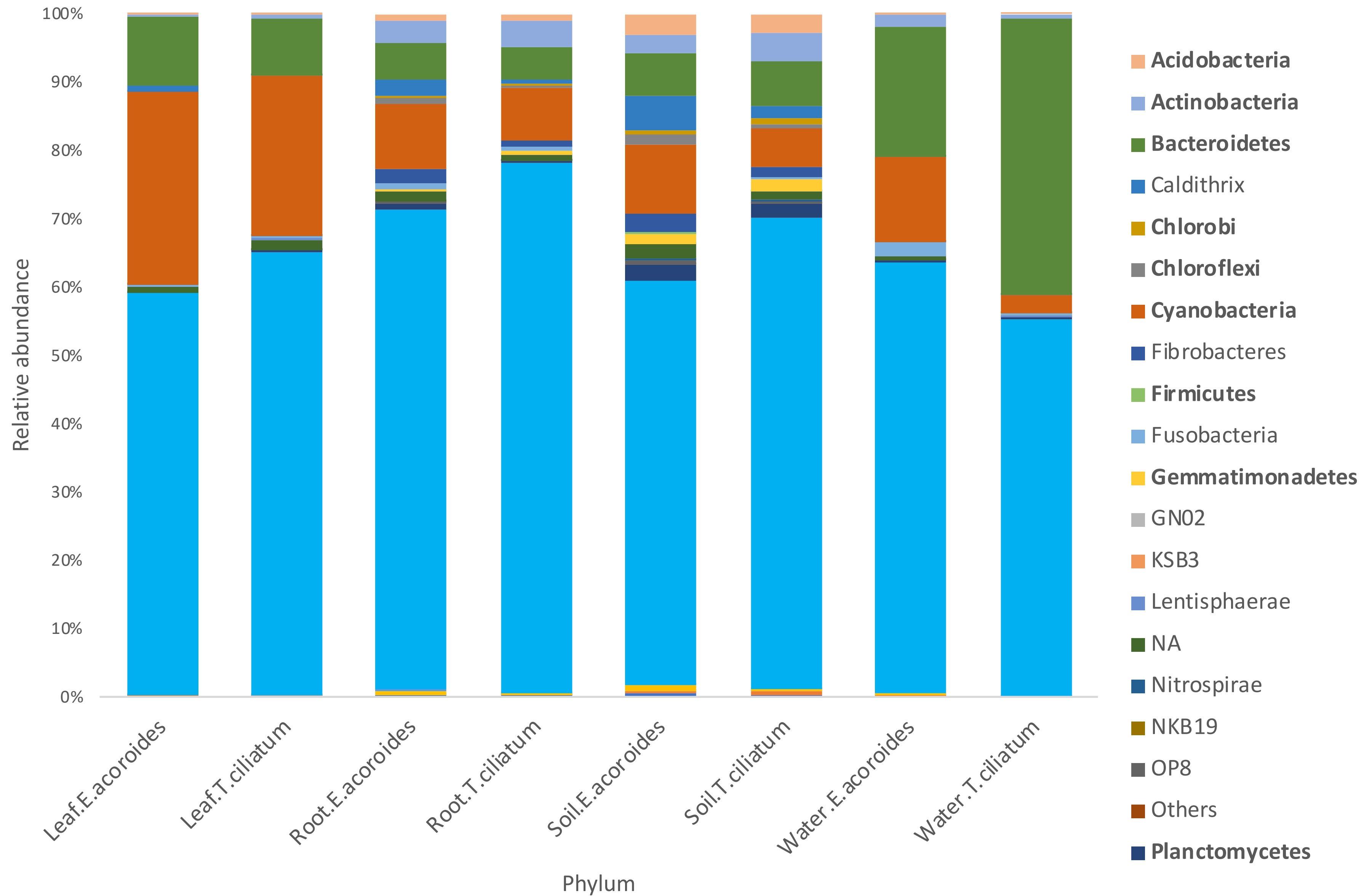

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Figure 5(on next page)

Venn diagram showing the core microbiome

(A) *T. ciliatum* and *E. acoroides* root and leaf OTUs clustered at similarity level of 97% is represented under each label. The number of unique and shared OTUs for each combination is shown. **(B)** Summary of taxonomic composition of the core microbiome and respective number of OTUs present in each phylum.

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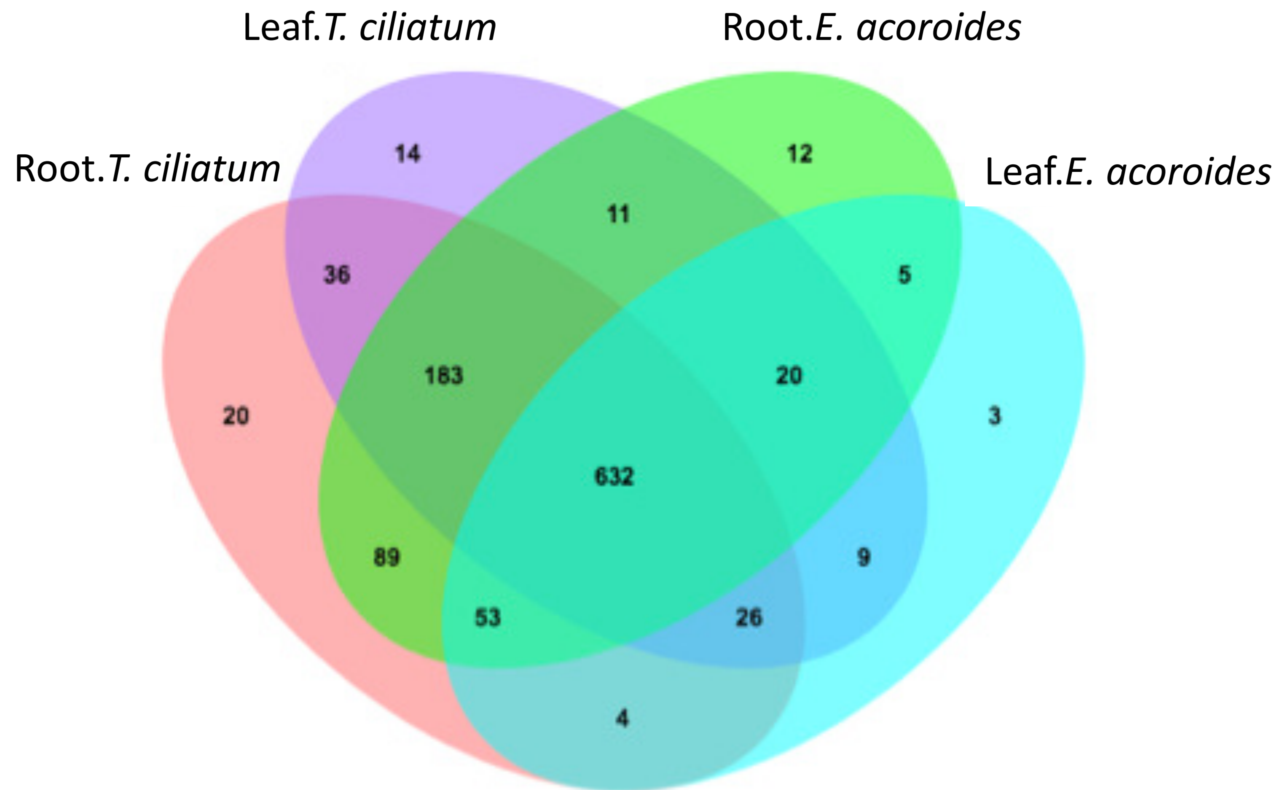
A



B

Phylum	#OTUs	%
Acidobacteria	20	3.16
Actinobacteria	14	2.22
Bacteroidetes	71	11.23
Caldithrix	8	1.27
Chlorobi	5	0.79
Chloroflexi	7	1.11
Cyanobacteria	29	4.59
Firmicutes	3	0.47
Fusobacteria	3	0.47
Gemmatimonadetes	8	1.27
GN02	2	0.32
GN03	1	0.16
GN04	1	0.16
Lentisphaerae	1	0.16
Nitrospirae	4	0.63
OP8	0	0.00
PAUC34f	1	0.16
Planctomycetes	26	4.11
Proteobacteria	410	64.87
SAR406	1	0.16
Spirochaetes	2	0.32
Thermi	1	0.16
Unassigned	7	1.11
Verrucomicrobia	2	0.32
WS3	5	0.79
Total	**632**	

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