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ORIGINAL ARTICLE

Remarkably Complex Microbial Community Composition in Bromeliad Tank Waters Revealed by eDNA Metabarcoding

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ABSTRACT

To investigate patterns of biotic community composition at different spatial scales and biological contexts, we used environmental DNA metabarcoding to characterize eukaryotic and prokaryotic assemblages present in the phytotelmata of three bromeliad species (*Aechmea gamosepala*, *Vriesea friburgensis*, and *Vriesea platynema*) at a single Atlantic Forest site in southern Brazil. We sampled multiple individuals per species and multiple tanks from each individual, totalizing 30 samples. We observed very high levels of diversity in these communities, and remarkable variation across individuals and even among tanks from the same individual. The alpha diversity was higher for prokaryotes than eukaryotes, especially for *A. gamosepala* and *V. platynema* samples. Some biotic components appeared to be species-specific, while most of the biota was shared among species, but varied substantially in frequency among samples. Interestingly, *V. friburgensis* communities (which were sampled at nearby locations) tended to be more heterogeneous across samples, for both eukaryotes and prokaryotes. The opposite was true for *V. platynema*, whose samples were more broadly spaced but whose communities were more similar to each other. Our results indicate that additional attention should be devoted to within-individual heterogeneity when assessing bromeliad phytotelmata biodiversity, and highlight the complexity of the biotic assemblages gathered in these unique habitats.

MICROBIAL communities across the world's ecosystems have been progressively shown to be highly variable and complex (Fenchel and Finlay 2004; Östman et al. 2010). While some habitat types have been extensively studied (Bettarel et al. 2003; Eilers et al. 2012), others remain vastly underexplored. This is the case of microhabitats such as phytotelmata, which are water bodies held in plant cavities, such as those created by modified leaves, leaf axils, flower bracts, tree holes, or bamboo internodes. These environments are widespread worldwide, comprise from small volumes to more than 45 liters (Frank and Lounibos 1987; Maguire 1971), and are likely to vary extensively in their biotic composition. Bromeliads (family Bromeliaceae) have a spiral arrangement of leaves, and in some species, the tight overlap of leaf-bases allows the formation of containers that accumulate rainwater and organic matter. This is an efficient strategy to store water and nutrients for subsequent absorption through foliar trichomes, especially for epiphytes and lithophytes (organisms with no soil supply).

The phytotelm microhabitat is known to harbor a large variety of bacteria, archaea, eukaryotes, and viruses (Goffredi et al. 2015; see also studies that focused on specific groups such as archaea [Brandt et al. 2015; Giongo et al. 2019], ciliates [Foissner et al. 2003; Simão et al. 2017], algae [Sophia et al. 2004], fungi [Sousa et al. 2014], Crustacea: [Jocque et al. 2013], and Arthropoda [Frank and Lounibos 2009]). Disease vectors of public health interest,

such as *Anopheles*, *Aedes*, and *Culex* mosquitoes (Frank and Lounibos 2009; Marques et al. 2012; Müller and Marcondes 2007) and Chagas disease vectors, that is, "kissing bugs" of the subfamily Triatominae (Bacigalupo et al. 2006; Simão et al. 2017), have also been reported in association with bromeliads. All these organisms jointly constitute a food web (Kitching 2000), with complex ecological interactions (e.g. predation, competition, detritophagy), most of which remain poorly characterized.

The colonization process of bromeliad tanks seems to be very complex, involving a series of interlinked events including active/passive dispersal, arrival, and establishment in an area (Maguire 1971). More than 50 years ago, Maguire (1963) (through experiments with artificial containers) pointed out three dispersal mechanisms into isolated bodies of water: wind and rain, flight, and animal assistance (phoresy). Years later, Frank and Lounibos (1987) compared the colonization process of phytotelmata with that of swamps and islands, differentiating these processes between phytotelm specialists with high species richness, and nonphytotelm specialists with low species richness. More recently, Dunthorn et al. (2012) conducted a study of diversity and endemism of ciliates inhabiting Neotropical phytotelmata (including bromeliads, bamboo, and tree holes) by sequencing the small subunit rDNA with the Sanger approach, coupled with morphological investigations. In that study, they described 45 isolates from phytotelmata, 28 of which consisted of species that are potentially endemic to this type of habitat. Thus, phytotelm communities would comprise both ubiquitous and endemic species, with some of the latter possibly being the result of speciation within this type of environment. Simão et al. (2017), using a metabarcoding approach to access phytotelmata diversity, concluded that ciliates and other unicellular eukaryotes were much more diverse than observed in studies based on morphology. These results suggest that these habitats harbor a hidden diversity that is difficult to survey without large-scale molecular assessments.

Due to the large environmental variation that bromeliad tanks are exposed to (mainly food restriction and hydric stress), organisms that inhabit phytotelmata can display survival mechanisms to escape life-threatening conditions. Such adaptations have been previously described for ciliates, including the formation of resistance cysts and shifts in cell size and oral ciliature enabling the change of dietary habits (Foissner et al. 2003). Other processes that may occur in phytotelm habitats are endospore formation (sporulation) in bacteria (Hutchison et al. 2014) and a phenomenon called cryptobiosis, which is an ametabolic (latent) state in response to hostile conditions (Keilin 1959). Anhydrobiosis is a kind of cryptobiosis induced by complete desiccation that promotes morphological, physiological, and molecular adaptations to reduce the effects of dehydration (Welnicz et al. 2011). This is a widespread phenomenon that has been found in tardigrades (water bears) (Halberg et al. 2013), rotifers (Marotta et al. 2010), and nematodes (Banton and Tunnacliffe 2012).

Previous studies on phytotelmata have revealed the high diversity of organisms that bromeliad tanks harbor, highlighting the need for more comprehensive assessments, including multiple samples per species and more powerful analytical tools. In addition, a study encompassing the characterization of prokaryotic and eukaryotic communities in bromeliad phytotelmata is still lacking. In this context, the main objectives of this study were to characterize and compare the prokaryotic and eukaryotic communities that live in phytotelmata formed by three bromeliad species sampled at a single location in the Brazilian Atlantic Forest, and to assess the heterogeneity in diversity patterns at three levels: (i) different bromeliad species; (ii) different individuals from the same species; and (iii) different tanks from the same individual. Our hierarchical sampling strategy allowed an unprecedented assessment of the complexity and spatial variation of these complex communities, serving as a baseline for in-depth investigations of their colonization process and functional dynamics.

MATERIALS AND METHODS

Sample collection

The study was carried out at the "Pro-Mata" Research Center ("Centro de Pesquisas e Conservação da Natureza Pró-Mata—PUCRS"), a private protected area located in the municipality of São Francisco de Paula, RS state, southern Brazil (29°27′–29°35′S; 50°08′–50°15′W) (Fig. S1). This area comprises ca. 3,100 hectares and is located on a high-elevation plateau (ca. 900 m asl), along with steep surrounding slopes (Fig. S1). It is mostly covered by "stricto sensu" Atlantic Forest vegetation (dense ombrophilous forest) and mixed ombrophilous forest (including *Araucaria* pines), as well as native grasslands and also a complex mosaic of vegetation at different stages of ecological regeneration after a period of logging prior to protection in the 1990s. In this area, it is possible to find two of the most abundant Bromeliaceae genera in the Brazilian Atlantic forest, *Vriesea* and *Aechmea* (Martinelli et al. 2008). The focal taxa of this study were *Aechmea gamosepala* Wittmack, *Vriesea friburgensis* Mez, and *Vriesea platynema* Gaud. These three species form water tanks and include epiphytic individuals. Samples were collected on the same day in Mach 2013, from three different tanks in each of three distinct epiphytic individuals (located between 1.5 and 2 m above the ground) of each species, totaling 27 samples. We selected three cisterns without direct communication, that is, the central cistern and two opposite-facing lateral cisterns. We also analyzed three additional samples (from different bromeliads), obtained from the central cistern of one individual of each species in a pilot sampling one month earlier (February, 2013). All the water stored in the selected cisterns was sampled by using a sterile pipette and preserved in an equal volume of TES lysis buffer (100 mM Tris, 100 mM EDTA, 2% SDS). Assuming that bromeliads have complex structures with large internal variation (tanks with different sizes and quantities of water and debris), our sampling design allowed us to investigate three levels of comparisons: (i) among species, (ii) among individuals of the

same species, and (iii) among cisterns of the same bromeliad individual.

The samples were named as follows: 3-letter code representing the species (AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*), the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns.

DNA extraction, amplification, and high-throughput sequencing

After homogenization of the sample, genomic DNA was extracted from a 250 μl aliquot using the DNeasy Blood & Tissue kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. For prokaryotes, we amplified the V4 region of the 16S rRNA gene using primers 515F and 806R (Bates et al. 2011), generating ~ 290-bp amplicons. For eukaryotes, we used primers targeting a 180–200 bp fragment of the 18S rRNA gene, including the V3 region (Nolte et al. 2010). The cycling conditions included an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 60 s, and a final extension step of 72 °C for 7 min. The solid-phase reversible immobilization (SPRI) paramagnetic bead technology (Agencourt AMPure XP; Beckman Coulter, Atlanta, GA) was used to purify the PCR amplicons. Library preparation was performed following the Ion Plus Fragment Library Kit manufacturer's recommendations (Thermo Fisher, Waltham, MA) from 100 ng of purified PCR products. The Ion Library Equalizer Kit (Thermo Fisher) and the Ion One Touch 2 system were used for normalization, template preparation, and enrichment of barcoded libraries. A multiplexed sequencing run was performed on an Ion PGM System (Thermo Fisher) using the Ion 316 Chip Kit v2. Low quality and polyclonal sequences were removed using default settings of the PGM software.

Analyses

Reads were trimmed to remove low-quality bases (Phred quality scores < 30) and short sequences using the software Prinseq (Schmieder and Edwards, 2011). Two different datasets based on minimum-length sequences were created to optimize the analyses of 16S and 18S rRNA. The first dataset contained sequences with size equal or greater than 100 bp, and the second one contained sequences equal or greater than 200 bp (high removal rate). The ≥ 100 bp dataset was used to assign DNA reads to taxonomic categories, while the ≥ 200 bp dataset was used to perform phylogenetic inferences, since with longer sequences it was possible to obtain a better alignment. We used USEARCH v7 (Edgar 2010) to merge identical reads (dereplication function), to discard unique sequences (singletons) so as to prevent the retention of sequencing errors, to check for chimeras, and to perform OTU clustering at a 99% similarity threshold.

For the prokaryote (16S rRNA) dataset, taxonomic assignment was performed with the UCLUST method and Greengenes v13_08 as the reference database. For the eukaryote (18S rRNA) dataset, we used the BLAST method and SILVA v111 as the reference database. Both analyses were conducted with QIIME v1.8.0 (Caporaso et al. 2010). The difference between the approaches employed for the two datasets was due to the better performance of the BLAST method with 18S rRNA data observed in pilot runs. We manually reviewed the assigned classifications, and, in case of doubtful assignments (e.g. marine groups), these taxa were assigned to reliable higher taxonomic levels. The ≥ 200 bp datasets were used to conduct sequence alignments, alignment filtering, and construction of phylogenetic trees, using the QIIME scripts *align_seqs.py*, *filter_alignment.py*, and *make_phylogeny.py*, respectively.

Before choosing the best method to statistically assess significant differences among groups of samples, Anderson–Darling and Shapiro–Wilk normality tests were performed with the Nortest package (http://www.inside-r.org/packages/cran/nortest). The QIIME script *group_significance.py* was used to compare OTU frequencies in sample groups to test for statistically significant differences between them. For the latter script, we use only the samples collected on the same day, so pilot samples (AGA01, VFR01, and VPL01) were excluded to avoid noise in the analyses. Using other packages implemented in R, we conducted additional analyses. The package Phyloseq (McMurdie and Holmes 2013) was used to calculate weighted UniFrac and unweighted UniFrac beta-diversity metrics, to conduct principal coordinates analysis (PCoA), and to estimate the alpha diversity through three measures: Chao1, Shannon, and Fisher. Graphical representations of phyloseq results were produced with the ggplot2 library (Wickham 2009). The Wilcoxon rank-sum test was used to compare the alpha-diversity measures between: (i) the entire prokaryotic community vs. the entire eukaryotic community, (ii) prokaryotic community vs. eukaryotic community of each bromeliad species, separately, and (iii) among different bromeliad species.

RESULTS

Both prokaryotic and eukaryotic communities were found to be remarkably diverse in these habitats. We observed high OTU richness, but only a few groups exhibited high abundance. The prokaryotic community was represented by 30 phyla or equivalent (candidate phyla). Proteobacteria was the most abundant phylum, corresponding in some cases to > 75% of the organisms found in a sample (Fig. 1). Of the 30 analyzed samples, only in one case (VPL4L1) was Proteobacteria not the most abundant group; in this sample, the dominant phylum was Bacteroidetes. At the genus level, the dominance of groups varied considerably. Looking at a subset with the 20 most abundant genera, only six phyla were observed. The genus with the greatest number of reads was *Pseudomonas* (Fig. 1), which displayed considerable variation in abundance across samples (see below).

The eukaryotic community was also highly diverse, with 67 identified phyla or equivalent. Ciliophora was the most

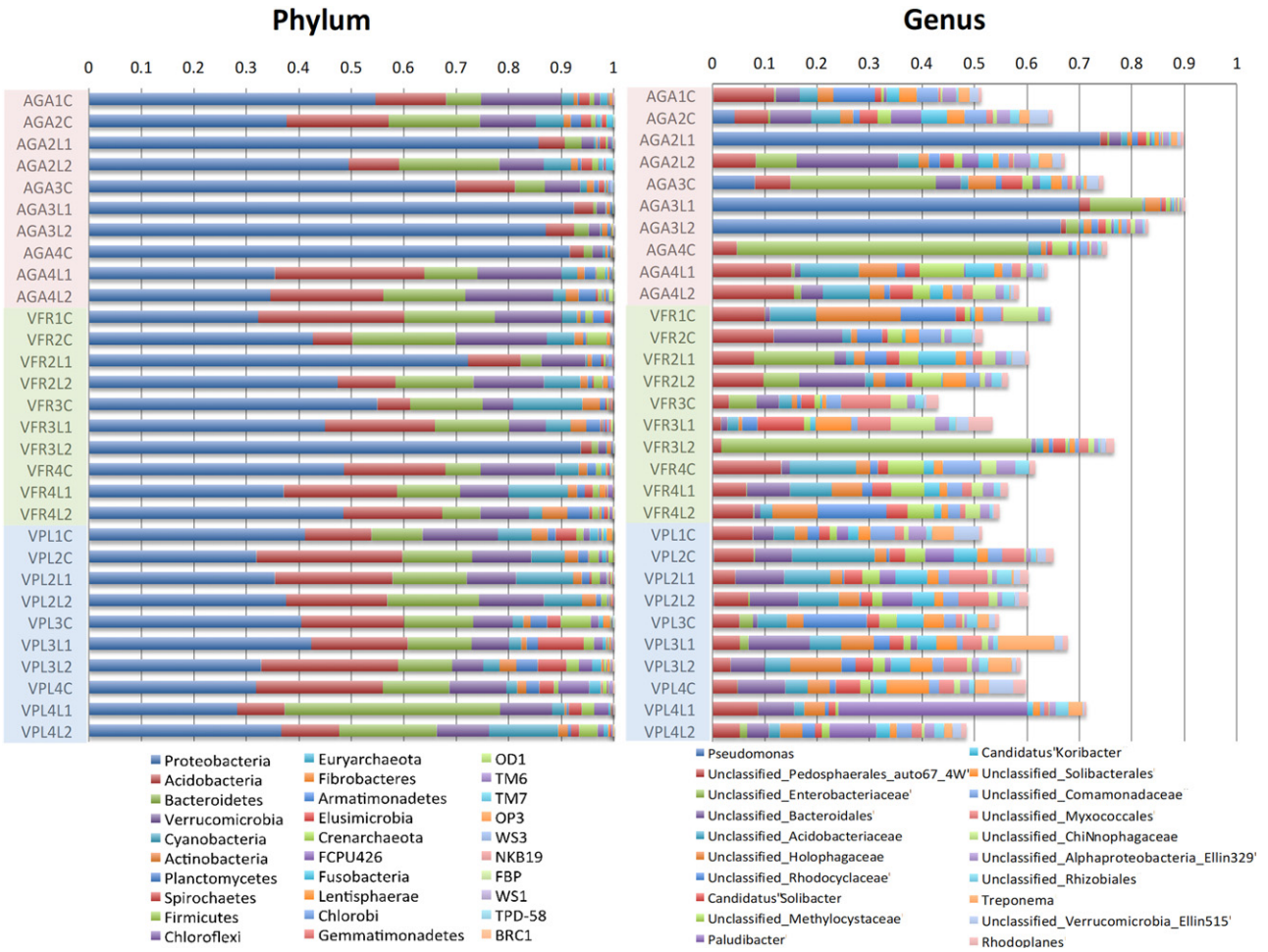

Figure 1 Relative abundance of prokaryotic phyla and genera in bromeliad tank water samples. The samples were named AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*, followed by the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns. Left: Relative abundance of the 30 prokaryotic phyla identified in this study. Right: Relative abundance of the top 20 prokaryotic genera identified in this study.

abundant phylum, followed by Arthropoda, Charophyta, Annelida, and Platyhelminthes (Fig. 2). At the genus level, reads assigned to "unclassified Ciliophora" were dominant, followed by "unclassified Naididae" (family of the phylum Annelida, formerly known as Tubificidae). The third and fourth places among the most abundant genera were *Glaucoma* and *Tetrahymena*, also belonging to phylum Ciliophora. In addition, approximately 1.5% of the classified reads were identified as belonging to genus *Anopheles*, probably from the species *Anopheles albimanus*. In one sample (VPL3L2), this genus represented more than 25% of the total reads (Fig. 2). *Culex* mosquitoes were identified as well, corresponding to 0.3% of the total reads. Finally, we found a few sequences belonging to genus *Trypanosoma*, representing ca. 0.1% of the reads, but sequences of its arthropod vectors were not detected. We also identified a variety of other organisms that likely play roles in the phytotelm ecological interaction network, such as ants (genus *Linepithema*), snails (e.g. Planorbidae gastropods, as well as the large marine and freshwater clade Caenogastropoda), and spiders (families Linyphiidae and Tetragnathidae).

The alpha-diversity results (Fig. 3 and Table 1) showed that species richness was higher for prokaryotes than eukaryotes, especially for *Vriesea platynema*, in which this pattern was consistent across all samples and the difference was significant with all three indices. The same consistent pattern (and significant differences overall) was observed for the Chao and Fisher indices (but not Shannon's) for *Aechmea gamosepala*. In contrast, the pattern was not observed for *Vriesea friburgensis*, with no consistent trend of prokaryotic diversity being higher than that of eukaryotes, and no significant difference in any of the three estimated indices (see Fig. 3 and Table 1). When alpha-diversity indices were compared among bromeliad species, all eukaryotic results (and almost all prokaryotic results) were nonsignificant (Table 1), indicating that all three species harbor similarly diverse biotas.

Even though their overall levels of diversity were similar, the three bromeliads presented some interesting

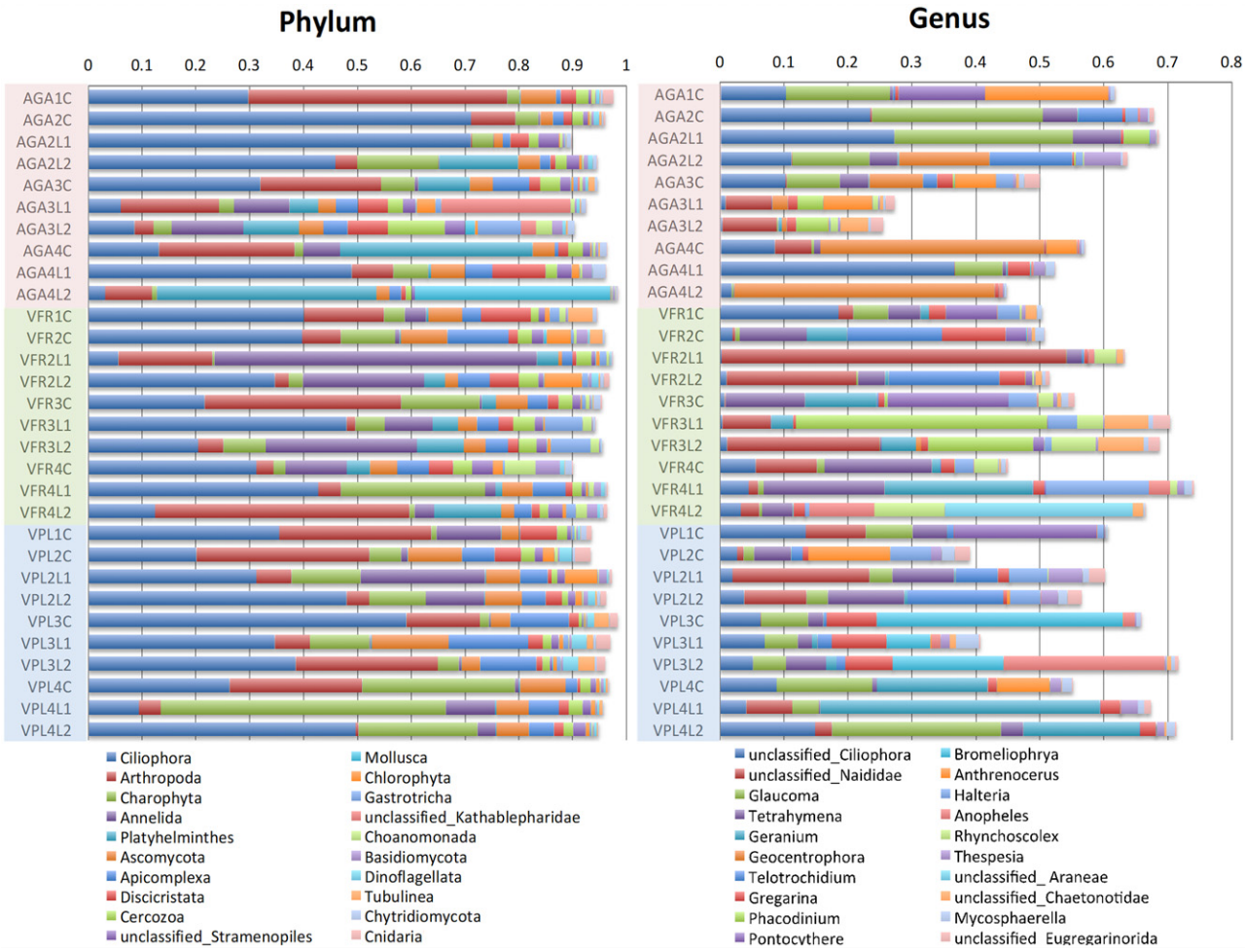

Figure 2 Relative abundance of the top 20 eukaryotic phyla (left) and genera (right) in bromeliad tank water samples. The samples were named AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*, followed by the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns.

differences with respect to community composition and intersample variation. We observed 71 prokaryotic and 37 eukaryotic OTUs whose frequencies were significantly different among bromeliad species (Tables 2 and 3). *Aechmea gamosepala* and *Vriesea friburgensis* each had 11 OTUs with significantly different frequencies relative to the two other species, while *Vriesea platynema* samples harbored 10 significantly different OTUs. Of these, 13 prokaryotic OTUs and 23 eukaryotic OTUs were found exclusively in one bromeliad species. Among prokaryotes, an interesting case was *Pseudomonas* (see Fig. 1), which was significantly more abundant in the tanks of *A. gamosepala* than in those of the other species (Table 2). Among eukaryotes, we found several cases of such significant differences, including *Neobodo designis* (four different OTUs, all found only in *A. gamosepala*), *Trypanosoma scelopori* (also found only in *A. gamosepala*), and the genus *Geocentrophora* (Platyhelminthes), which was represented by seven OTUs detected almost exclusively in *A. gamosepala* tanks (see Table 3). Such findings suggest that some of components of the phytotelm community may be host-specific.

Overall differences in species composition could be assessed more broadly with the PCoA plots. When we used unweighted UniFrac distances for the prokaryotic community, we observed two clusters (Fig. 4), one of which comprised all *V. friburgensis* samples, while the other comprised the biotas sampled in the other two species. Interestingly, *V. friburgensis* was not only distinct from the other two species, but also presented a more prominent intersample variability, especially along axis two, while *V. platynema* showed a more restricted distribution on both axes. Most *A. gamosepala* samples clustered together, but some of them clustered closely with *V. platynema* samples. Finally, it was noteworthy that one of the *V. friburgensis* individuals (VFR3) presented samples (central and both lateral) that clustered together, a pattern that was not apparent in the samples from the other species. These patterns were less visible when we used weighted UniFrac distances (Fig. 4, bottom),

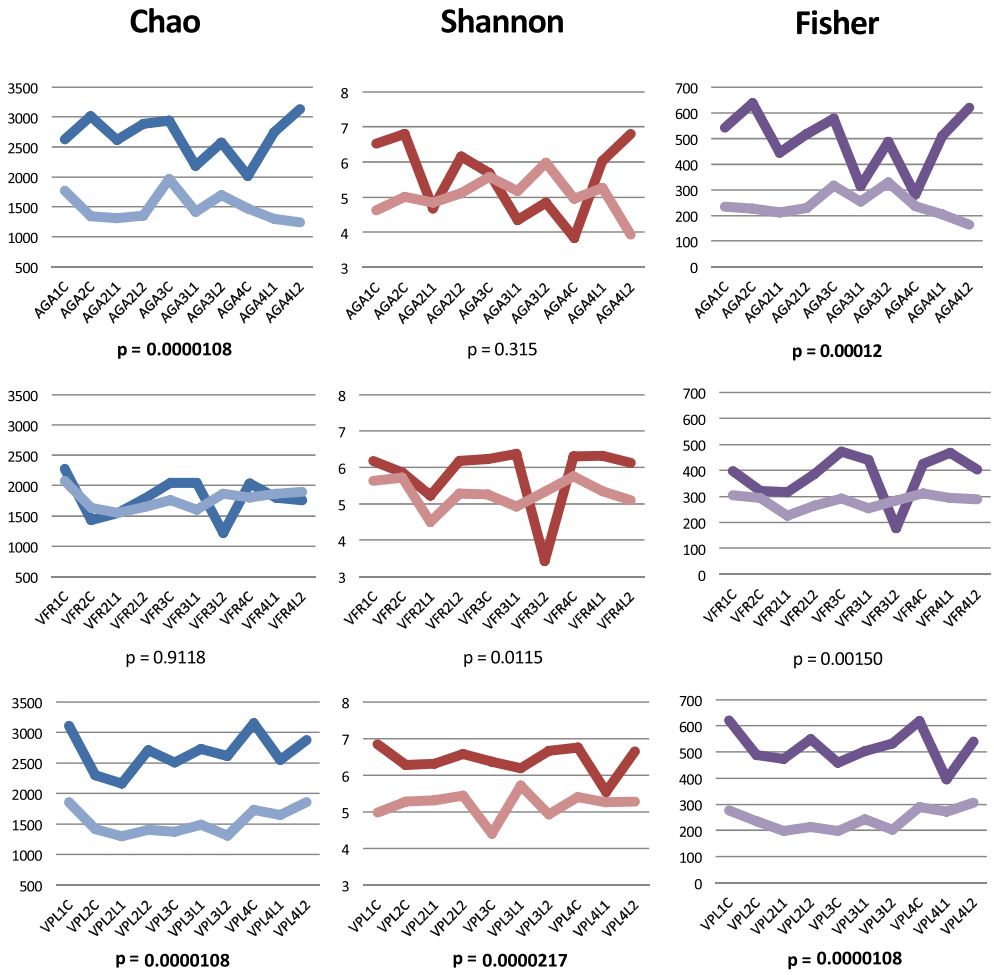

Figure 3 Alpha-diversity measures of bromeliad tank water communities. Wilcoxon rank-sum test *P*-values are shown below each chart. The samples were named AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*, followed by the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns.

Table 1. Results of Wilcoxon rank-sum test for alpha-diversity measures in eukaryotic and prokaryotic communities contained in phytotelmata of three different bromeliad species

Chao 1	*P*-value	Shannon	*P*-value	Fisher	*P*-value
16S vs. 18S (all spp.)	**<0.000001**	16S vs. 18S (all spp.)	**<0.000001**	16S vs. 18S (all spp.)	**<0.000001**
AGA16S vs. AGA18S	**<0.000001**	AGA16S vs. AGA18S	0.315	AGA16S vs. AGA18S	**0.0001299**
VFR16S vs. VFR118S	0.9118	VFR16S vs. VFR18S	0.0115	VFR16S vs. VFR18S	0.001505
VPL16S vs. VPL18S	**<0.000001**	VPL16S vs. VPL18S	**<0.000001**	VPL16S vs. VPL18S	**<0.000001**
AGA16S vs. VFR16S	**0.0002057**	AGA16 vs. VFR16S	0.6305	AGA16 vs. VFR16S	0.02323
AGA16S vs. VPL16S	0.7394	AGA16S vs. VPL16S	0.07526	AGA16S vs. VPL16S	0.918
VFR16S vs. VPL16S	**<0.000001**	VFR16S vs. VPL16S	0.0115	VFR16S vs. VPL16S	0.00105
AGA18S vs. VFR18S	0.01469	AGA18S vs. VFR18S	0.2176	AGA18S vs. VFR18S	0.07526
AGA18S vs. VPL18S	0.5787	AGA18S vs. VPL18S	0.315	AGA18S vs. VPL18S	0.9705
VFR18S vs. VPL18S	0.02323	VFR18S vs. VPL18S	0.5787	VFR18S vs. VPL18S	0.03546

Significantly different values ($P \leq 0.001$) are shown in bold.

although the lower intersample diversity of *V. platynema* relative to the other species was still apparent, especially on axis 1 (which explained 50% of the overall variance).

The PCoA plots of the eukaryotic community revealed some patterns that were remarkably similar to those observed in prokaryotes (Fig. 5). With unweighted UniFrac

Table 2. Operational taxonomic units (OTUs) whose abundance was significantly different among the prokaryotic communities of the three bromeliad species surveyed here

AGA	VFR	VPL	*P*-value	Taxonomic assignment		
3	0	18	0.00013	Acidobacteria	Acidobacteriaceae	
32	3	33	0.00064	Acidobacteria	Acidobacteriaceae	
2	0	11	0.00065	Acidobacteria	Acidobacteriaceae	
46	0	0	0.00068	Acidobacteria	Acidobacteriaceae	
6	0	0	0.00069	Acidobacteria	Acidobacteriaceae	
11	2	51	0.00009	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
9	2	24	0.00014	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
5	0	10	0.00055	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
32	0	3	0.00082	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
3	1	18	0.00087	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
2	0	20	0.00092	Acidobacteria	Koribacteraceae	Candidatus *Koribacter*
24	0	5	0.0007	Acidobacteria	Holophagales	Holophagaceae
5	3	21	0.00097	Acidobacteria	Holophagales	Holophagaceae
37	1	148	0.00009	Acidobacteria	Solibacterales	
7	1	34	0.00026	Acidobacteria	Solibacterales	
4	1	20	0.00051	Acidobacteria	Solibacterales	
6	0	19	0.00051	Acidobacteria	Solibacterales	
4	0	17	0.00057	Acidobacteria	Solibacterales	
0	0	3	0.00069	Acidobacteria	Solibacterales	
1	0	4	0.00037	Acidobacteria	Solibacteraceae	Candidatus *Solibacter*
11	2	16	0.00074	Acidobacteria	Solibacteraceae	Candidatus *Solibacter*
1	1	5	0.00066	Actinobacteria	Actinomycetales	Microbacteriaceae
0	21	104	0.00043	Bacteroidetes		
0	0	7	0.00032	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
2	0	84	0.00034	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
25	0	277	0.00035	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
77	0	365	0.00052	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
9	0	78	0.00063	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
2	0	14	0.00076	Bacteroidetes	Porphyromonadaceae	*Paludibacter*
2	0	3	0.00071	Bacteroidetes	Cytophagales	*Cytophaga*
0	0	8	0.00014	Chloroflexi	Anaerolineales	Anaerolinaceae
38	1	442	0.0002	Chloroflexi	Anaerolineales	Anaerolinaceae
36	0	75	0.00022	Cyanobacteria	Streptophyta	
73	5	96	0.00054	Cyanobacteria	Streptophyta	
4	0	21	0.0007	Euryarchaeota	Methanosarcinaceae	*Methanosarcina*
32	1	44	0.00045	Euryarchaeota	E2	Methanomassiliicoccaceae
1	0	2	0.00066	Firmicutes	Clostridiales	Christensenellaceae
2	0	6	0.0003	Firmicutes	Clostridiales	Ruminococcaceae
6	2	14	0.00083	Firmicutes	Clostridiales	Ruminococcaceae
2	1	10	0.00085	Firmicutes	Clostridiales	*Ethanoligenens*
3	1	10	0.00081	Firmicutes	Clostridiales	Veillonellaceae
25	0	0	0.00014	OD1	SM2F11	
9	1	39	0.00007	Planctomycetes	Pirellulales	Pirellulaceae
3	0	9	0.00043	Planctomycetes	Pirellulales	Pirellulaceae
1	0	9	0.00053	Planctomycetes	Pirellulales	Pirellulaceae
1	1	9	0.00098	Planctomycetes	Pirellulales	Pirellulaceae
5	0	0	0.00004	Planctomycetes	Planctomycetaceae	*Planctomyces*
1	0	4	0.00028	Proteobacteria	Hyphomicrobiaceae	
7	4	28	0.00038	Proteobacteria	Hyphomicrobiaceae	*Rhodoplanes*
4	0	5	0.00028	Proteobacteria	Methylocystaceae	
14	1	25	0.00052	Proteobacteria	Rhizobiaceae	
7	0	10	0.00019	Proteobacteria	Rhizobiaceae	*Rhizobium leguminosum*
7	0	9	0.00042	Proteobacteria	Rhizobiaceae	*Rhizobium leguminosum*
8	1	9	0.00095	Proteobacteria	Sphingomonadaceae	
2	0	3	0.00042	Proteobacteria	Sphingomonadaceae	*Sphingobium*

(continued)

Table 2. (continued)

AGA	VFR	VPL	*P*-value	Taxonomic assignment		
9	2	45	0.00034	Proteobacteria	Rhodocyclaceae	*Uliginosibacterium*
4	1	22	0.00072	Proteobacteria	Rhodocyclaceae	*Uliginosibacterium*
13	0	0	0.00068	Proteobacteria	SC-I-84	
7	0	86	0.00003	Proteobacteria	Myxococcales	
8	0	68	0.00006	Proteobacteria	Myxococcales	
4	0	0	0.00068	Proteobacteria	Myxococcales	
0	0	18	0.00002	Proteobacteria	Myxococcaceae	*Anaeromyxobacter*
18	17	81	0.00085	Proteobacteria	Myxococcaceae	*Anaeromyxobacter*
4	0	27	0.00011	Proteobacteria	Syntrophobacteraceae	*Syntrophobacter*
603	0	3	0.00013	Proteobacteria	Pseudomonadaceae	*Pseudomonas*
100	0	0	0.00048	Proteobacteria	Pseudomonadaceae	*Pseudomonas*
1697	0	5	0.00054	Proteobacteria	Pseudomonadaceae	*Pseudomonas*
108	0	0	0.00061	Proteobacteria	Pseudomonadaceae	*Pseudomonas*
33	0	0	0.00069	Proteobacteria	Pseudomonadaceae	*Pseudomonas*
4	1	17	0.00038	Verrucomicrobia	Pedosphaerales	auto67_4W
6	1	29	0.00049	Verrucomicrobia	Pedosphaerales	auto67_4W

The first three columns show values of OTU abundance (in number of reads) for each bromeliad species (AGA: *Aechmaea gamosepala*; VFR: *Vriesea friburgensis*; VPL: *Vriesea platynema*). The other columns indicate the *P*-value from a Kruskal–Wallis test comparing abundance among species, and the taxonomic assignment of the OTU.

distances, we also observed two major clusters (although here they were separated along axis 2), one of which comprised all *V. friburgensis* samples, while the other comprised *A. gamosepala* and *V. platynema*. In addition, *V. friburgensis* presented the largest intersample variability and *V. platynema* the smallest. At the same time, there was an interesting difference relative to the prokaryotic results, pertaining to community variation among cisterns of the same individual. With eukaryotes, although within-individual differences were still large, it was more common to observe nonoverlapping clusters formed by samples from each bromeliad specimen. As in prokaryotes, this trend was stronger in *V. friburgensis* (all of whose individuals formed nonoverlapping clusters comprising their three cisterns), but here it could also be observed with *V. platynema* (e.g. VPL3) and *A. gamosepala* (e.g. AGA3). Finally, we also observed that these patterns were lost when we employed weighted UniFrac distances (Fig. 5, bottom), similar to what was seen with prokaryotes.

DISCUSSION

Very high levels of eukaryotic and prokaryotic diversity were observed in all the analyzed samples. When all the samples are considered jointly, 30 different prokaryotic phyla and 67 eukaryotic phyla were detected in this system. Considering the spatial scale of our sampling (see Fig. S1), and the fact that we targeted bromeliads in similar ecological contexts (same elevation, same type of vegetation, all epiphytes at similar heights above the ground), such a diversity is remarkable, and likely represents an underestimate of what may be found when further variation in these ecological parameters is explored. This rationale is supported by the observations reported by Richardson (1999), who studied three different forest types at two different elevations in Puerto Rico, and observed that the overall diversity of invertebrates in bromeliad tanks was lowest in a forest composed by plants of a small size. Also, the alpha diversity in the phytotelmata decreased with increasing elevation. Other abiotic factors could also influence the abundance and diversity of organisms present in bromeliad tanks, as demonstrated in other studies (e.g. Goffredi et al. 2011).

Since phytotelmata are located within terrestrial or semiterrestrial ecosystems such as forests, woodlands, and swamps (Kitching 2000), and contain freshwater and mud strata, their aquatic communities should comprise a mixture of typical freshwater and soil taxa. On the other hand, the small size and physiological fluctuations imposed to the organisms by these environments might lead to colonization by very specialized communities (Kitching 1971; Yanoviak 1999). We observed the three types of colonists in the assessed phytotelmata, including a flatworm species previously reported from soil samples (*Geocentrophora sphyrocephala*) (Adl et al. 2006) and ciliates that represent both common limnetic (e.g. *Glaucoma*, *Halteria*) and bromeliad-specialist (*Bromeliophrya*) taxa (see Fig. 2 and Table 3).

As reported in other studies, Ciliophora was the most abundant eukaryotic phylum observed during the sampling period, reaching a relative abundance of ca. 70% in two bromeliad cisterns (Fig. 2). Annelids and arthropods were also very abundant (ca. 60% and 50%, respectively, in particular samples) and were present in all three bromeliad species. Apicomplexa was also present as one of the 20 most abundant phyla, probably due to its frequent association with invertebrates in the phyla Arthropoda and Annelida. Interestingly, genus *Gregarina*, a group that commonly parasitizes annelids and arthropods, was observed here to

Table 3. Operational taxonomic units (OTUs) whose abundance was significantly different among the eukaryotic communities of the three bromeliad species surveyed here

AGA	VFR	VPL	*P*-value	Taxonomy		
0	0	3	0.00016	Amoebozoa	Arcellinida	*Cryptodifflugia operculata*
1	11	567	0.00018	Amoebozoa	Euamoebida	*Tubulinida* sp.
29	10	177	0.00082	Streptophyta	Lamiales	*Vitex cofassus*
20	1	49	0.00081	Streptophyta	Liliopsida	*Phaseolus acutifolius*
113	9	241	0.00045	Streptophyta	Liliopsida	*Phaseolus acutifolius*
134	0	0	0.00069	Euglenozoa	Neobodonida	*Neobodo designis*
256	0	0	0.0007	Euglenozoa	Neobodonida	*Neobodo designis*
58	0	0	0.00097	Euglenozoa	Neobodonida	*Neobodo designis*
18	0	0	0.00098	Euglenozoa	Neobodonida	*Neobodo designis*
552	2	0	0.00056	Euglenozoa	Neobodonida	*Rhynchomonas nasuta*
0	1	88	0.00008	Euglenozoa	Trypanosomatida	*Strigomonas galati*
0	0	7	0.00026	Euglenozoa	Trypanosomatida	*Strigomonas galati*
105	0	0	0.00098	Euglenozoa	Trypanosomatida	*Trypanosoma scelopori*
0	4	0	0.00098	Fungi	Ascomycota	*Mycosphaerellaceae*
13	11	40	0.00078	Fungi	Ascomycota	*Sarcinomyces* sp.
0	9	0	0.00022	Fungi	Chytridiomycota	*Kappamyces laurelensis*
21	0	0	0.00098	Fungi	Kickxellomycotina	*Orphella haysii*
0	1	108	0.00004	Fungi	unclassified	Fungi
0	1	31	0.00007	Mollusca	unclassified	Mollusca
0	9	0	0.00059	Platyhelminthes	Catenulida	*Rhynchoscolex simplex*
0	3	0	0.00077	Platyhelminthes	Catenulida	*Rhynchoscolex simplex*
0	6	0	0.00077	Platyhelminthes	Catenulida	*Rhynchoscolex simplex*
2	88	1	0.00094	Platyhelminthes	Catenulida	*Rhynchoscolex simplex*
299	1	0	0.00071	Platyhelminthes	Lecithoepitheliata	*Geocentrophora* sp.
10	0	0	0.00098	Platyhelminthes	Lecithoepitheliata	*Geocentrophora* sp.
32	0	0	0.00071	Platyhelminthes	Lecithoepitheliata	*Geocentrophora sphyrocephala*
28	0	0	0.00077	Platyhelminthes	Lecithoepitheliata	*Geocentrophora sphyrocephala*
53	1	0	0.00089	Platyhelminthes	Lecithoepitheliata	*Geocentrophora sphyrocephala*
35	0	0	0.00098	Platyhelminthes	Lecithoepitheliata	*Geocentrophora sphyrocephala*
17	0	0	0.00098	Platyhelminthes	Lecithoepitheliata	*Geocentrophora sphyrocephala*
1	2	10	0.00063	SAR	Apicomplexa	*Gregarina*
0	0	71	0.00004	SAR	Apicomplexa	*Apicystis*
0	31	0	0.00021	SAR	Ciliophora	*Loxophyllum*
1	0	27	0.00008	SAR	Dinoflagellata	Dinophyceae
3	0	0	0.00098	SAR	Stramenopiles	MAST-12
5	0	0	0.00022	SAR	Stramenopiles	MAST-3
37	0	0	0.00022	SAR	Stramenopiles	MAST-3

The first three columns show values of OTU abundance (in number of reads) for each bromeliad species (AGA: *Aechmaea gamosepala*; VFR: *Vriesea friburgensis*; VPL: *Vriesea platynema*). The other columns indicate the *P*-value from a Kruskal–Wallis test comparing abundance among species, and the taxonomic assignment of the OTU.

rank among the top 20 most abundant genera. A similar pattern was observed in a previous study in the same area (Simão et al. 2017), indicating a consistent trend. Still, it is noteworthy that the presence and abundance of these groups were considerably variable across samples, even when comparing cisterns from the same bromeliad individual sampled on the same day (see Fig. 2).

In phytotelmata, studies have shown that many organisms may be bromeliad specialists, not being found in other types of habitat (Benzing 2000). With respect to ciliates, so far at least three new genera and *ca.* 10 new species were described as bromeliad-water endemics (Foissner 2003; Foissner et al. 2003). Here, we were able to identify groups of ciliates that have been found only in bromeliad water, such as *Bromeliophrya brasiliensis* (Foissner 2003) and *Platyophrya bromelicola* (Foissner and Wolf 2009). Relative abundance data revealed that the genus *Bromeliophrya* was present in *V. platymena*, reaching ca. 40% of the analyzed OTUs in one sample (VPL3C) (Fig. 2).

On the other hand, there are common freshwater species that are found in bromeliad water. For example, *Halteria grandinella*, an abundant species in freshwater environments, was observed in bromeliads from Ecuador (Katz et al. 2005) and also reported at high densities in bromeliads from the upper Paraná River (Buosi et al. 2015). In that same study, the scuticociliate *Cyclidium glaucoma* was also found at high abundance. High

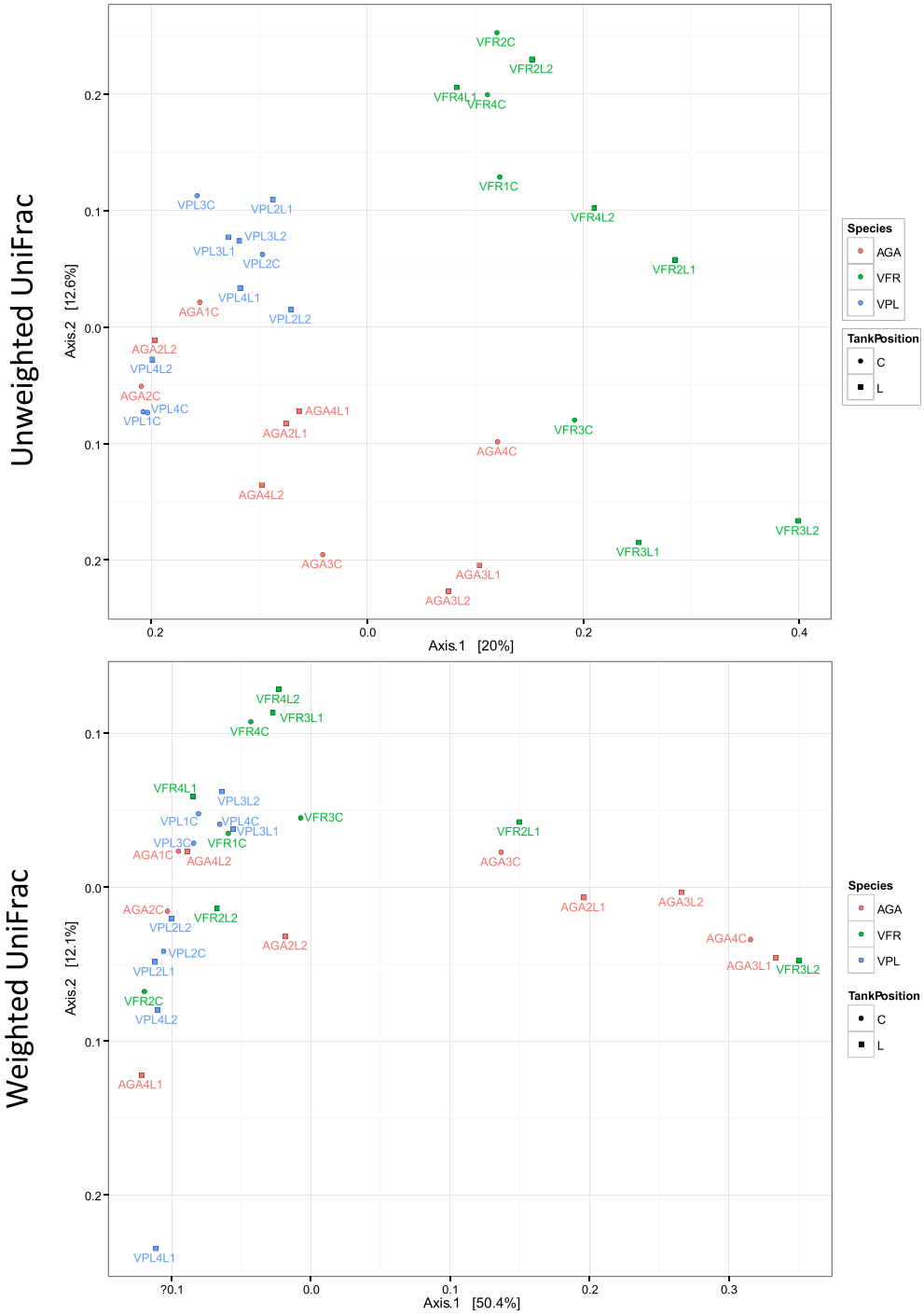

Figure 4 Principal coordinate analysis (PCoA) plot of the sampled prokaryotic communities based on Unweighted (top) and Weighted (bottom) UniFrac distances. The samples were named AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*, followed by the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns.

densities of scuticociliates are reported from freshwater environments with high amounts of nutrients, similar to bromeliad phytotelmata. Although species reported for lakes and rivers have been found in bromeliad waters, some very common species in these environments lack a record for phytotelmata. For example, the genus *Paramecium* was reported from the water-filled bracts of *Heliconia caribea* from Porto Rico, but was never collected from bromeliad water. Dunthorn et al. (2012), in a phylogenetic study of ciliates from bromeliad water collected in Jamaica

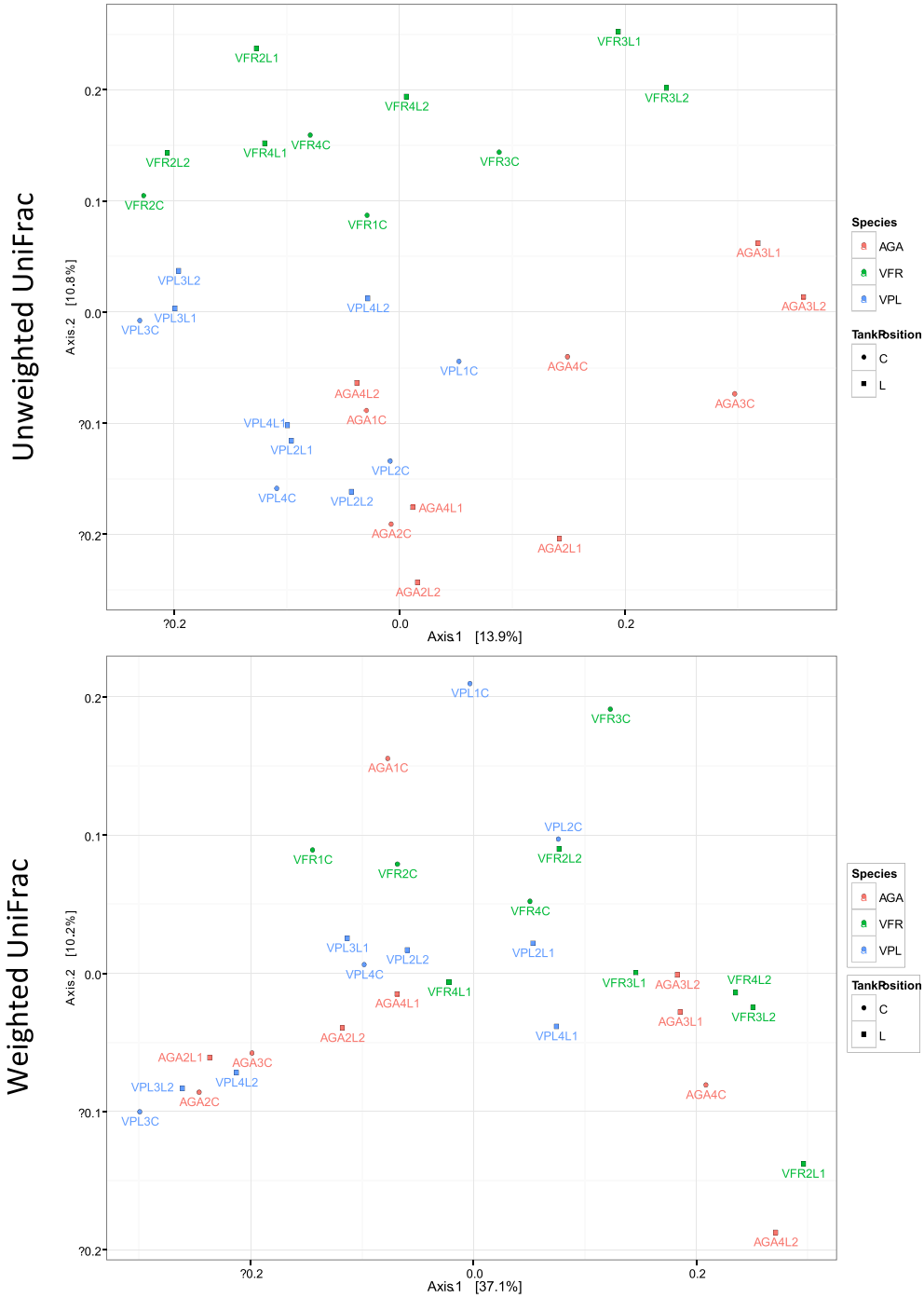

Figure 5 Principal coordinate analysis (PCoA) plot of the sampled eukaryotic communities based on Unweighted (top) and Weighted (bottom) UniFrac distances. The samples were named AGA for *Aechmea gamosepala*, VFR for *Vriesea friburgensis*, and VPL for *Vriesea platynema*, followed by the individual number and the tank position, where the letter C represents the central cistern and L1/L2 represent the two lateral cisterns.

and in Brazil, pointed out that the genus *Paramecium* was never observed in a sample of over 200 bromeliads. The first record of *Paramecium* in bromeliad water was made by Buosi et al. (2015) for *Aechmaea disticantha* from the Upper Paraná River in Brazil. In that study, *Paramecium multimicronucleatum* was present only in bromeliads that received river water during the flood, which led to the inference that the river was the source of these ciliates. Vendermeer et al. (1972) experimentally inoculated *Paramecium* cultures in bromeliads from Costa Rica in

different laboratory conditions, and concluded that the interaction between *Paramecium* and the microcommunity in the bromeliads led to competitive exclusion of the former. Interestingly, here we were able to detect the presence of *Paramecium* in all sampled bromeliad species. Since the bromeliads sampled here are far from any freshwater source, we can conclude that the detected *Paramecium* individuals were brought by other dispersal agents. For example, Maguire and Belk (1967) demonstrated that terrestrial snails in the genus *Caracolous* transported *Paramecium multimicronucleatum* among *Heliconia* flowers. Therefore, in case *Paramecium* populations go extinct in a given bromeliad tank, such dispersal could facilitate recolonization from nearby sources.

In addition to *Paramecium*, common freshwater genera such as *Glaucoma*, *Coleps*, *Frontonia*, *Colpidium*, *Nassula*, *Stylonichia*, and *Trithigmostoma* had also not been recorded previously in bromeliad waters (Dunthorn et al. 2012). Here, we detected sequences belonging to the genera *Glaucoma*, *Coleps*, *Trithigmostoma*, and *Frontonia*, confirming their presence in bromeliad water. Interestingly, the genus *Glaucoma* was also found at high abundance (*ca*. 20%) and was sampled from the three species of bromeliads. In general, hymenostome ciliates (subclass Hymenostomatia) generally present high diversity in phytotelmata. Dunthorn et al. (2012) isolated nine species of hymenostomes from bromeliad waters, the highest richness among ciliate subclasses in that study. Likewise, Buosi et al. (2015) reported that hymenostomes represented 22% of the total abundance of ciliates in bromeliads from the upper Paraná River. Simão et al. (2017) also reported high abundances of *Glaucoma* and *Tetrahymena* in the cisterns of *Aechmaea gamosepala* and *Vriesea platynema*. A similar result was observed here, with the hymenostome genus *Tetrahymena* being one of the most abundant unicellular organisms observed in the sampled bromeliads (Fig. 2).

To better understand the ecological context in which these eukaryotic communities thrive, we also investigated the prokaryotic components that comprise these biotic assemblages. Maguire (1971) pointed out the abundance of prokaryotes involved in the decomposition of accumulated material in plant-held waters, while Pittl et al. (2010) suggested that bacteria present in bromeliad water are capable of decomposing plant material as well as chitin, the main component of arthropod skeletons. In spite of these initial efforts, analyses of the composition and dynamics of the prokaryotic communities present in these habitats are in their infancy, and very little is known regarding their interactions with the eukaryotic community.

Several authors have reported a high diversity of Bacteria and Archaea in bromeliad tank waters. For example, Goffredi et al. (2015), using transcriptomic analyses, found genes belonging to 25 different bacteria phyla and to 7 different archaeal orders in one bromeliad species from Costa Rica. The ten most highly expressed genes were from organisms belonging to the phyla Spirochaetes, Acidobacteria, Firmicutes, and Euryarchaeota. Fluctuations of the bacterial and archaeal communities have also been linked to environmental factors. For example, Goffredi et al. (2011), analyzing the prokaryotic community in bromeliads from Costa Rica, observed that in more acidic bromeliads the composition of the prokaryotic community is more similar to peat bogs than to the surrounding soil, suggesting that the bacterial diversity may be governed by the acid-base fluctuation of the water. Several other factors are likely to drive prokaryotic community composition in these systems, including interactions with the plant itself. For example, Giongo et al. (2019) observed that *Pseudomonas* and *Enterobacter* (both of which have been reported to contain plant-beneficial species) were the two most abundant bacterial genera found in *Aechmaea gamosepala* and *Vriesea platynema* at the same Atlantic Forest site investigated here.

Here, Proteobacteria dominated the prokaryotic community of the three sampled species during the whole study period. Several studies have demonstrated the presence of Proteobacteria in bromeliad water (Goffredi et al. 2011, 2015; Lehours et al. 2010) and associated it with acidic conditions and degradation. Klan et al. (2016) observed that Betaproteobacteria were the most abundant bacterial group in one species of bromeliad from Costa Rica. According to the metabolic profiles obtained, the authors suggested that this group of bacteria plays an essential role in the break-down of plant source material within the bromeliad tank. Other abundant groups, such as Acidobacteria, Verrucomicrobia, and Firmicutes, have also been reported from bromeliad water (Goffredi et al. 2015; Goffredi et al. 2011; Louca et al. 2017), generally associated with changes in pH due to the decomposition of plant organic matter.

The metazoan fauna present in bromeliad tanks has been known since the beginning of the 20th century. Picado (1913) published a pioneering study on organisms inhabiting bromeliad tank water. In that study, he pointed out the presence of insects in bromeliad tanks and suggested that the colonization of the bromeliads was driven by stochastic events such as wind, rain, passive transportation, and flight. Laessle (1961), in a study of bromeliads from Jamaica, found insect larvae as well as copepods and ostracods inhabiting bromeliad tank water. Other studies have reported the presence of ants, spiders, isopods, and oligochaetes composing the metazoan community in phytotelmata of different bromeliad species (Hénaut et al. 2014; Leroy et al. 2013; Richardson 1999).

Here, in addition to Arthropoda and Annelida, Platyhelminthes was among the 20 most abundant phyla. The genera *Geocentrophora* and *Rhynchoscolex* presented relative abundances of *ca*. 40% in some bromeliad individuals. Free-living platyhelminthes have been reported inhabiting bromeliad waters. For example, Céréghino et al. (2018) observed that flatworms composed 22% of the nonarthropod metazoan fauna of bromeliads located in 10 different countries in the Neotropical region, demonstrating that these metazoans could be a considerable portion of the phytotelm community. Here, we also detected tardigrades, rotifers, and nematodes. These groups have

some anhydrobiotic members (Watanabe 2006) that are capable to survive for years in a dormant state; in the case of rotifers, we identified reads belonging to the *Mniobia* genus, being some of them probably from the species *Mniobia russeola*. This species was reported as an anhydrobiotic organism, along with other representatives of the same genus. For nematodes, we observed the presence of *Tylenchus* and *Ditylenchus*, also reported to potentially undergo anhydrobiosis. Our tardigrade sequences were classified as *Diphascon*, *Halobiotus*, and *Isohypsibius*, belonging to the family Hypsibiidae, and *Macrobiotus* and *Minibiotus*, members of family Macrobiotidae. Both families have at least one reported anhydrobiotic species (*Ramazzottius oberhaeuseri* and *Adorybiotus coronifer*, respectively). The presence of close relatives of organisms that have been previously described as resistant to desiccation indicates that the anhydrobiosis phenomenon may occur in the phytotelm environment, and may be important for the persistence of these organisms in the environment during a period of drought.

Considering the fragmentation of phytotelm environments, the invasion and colonization processes of isolated bodies of water (here represented by each bromeliad or each bromeliad tank) by small aquatic organisms can be compared to dispersal mechanisms operating in oceanic islands, where smaller isolation distances result in more frequent successful events of colonization (Maguire 1963). Thus, it would be expected that the closest bromeliads would be more similar to each other. However, colonization processes depend on local abiotic and biotic factors that will determine the success of the species that arrived in the environment (Ricklefs 1987). Many microorganisms are able to make cysts that are easily dispersed by the wind and could survive harsh environmental conditions (Corliss and Esser 1974), while others can disperse by small droplets of water, or via insects or birds (Foissner 2006). Thus, dispersal may be a determining factor by which organisms can reach long distances and colonize environments on a broad scale (Maguire 1963). Several studies have demonstrated that shaping of communities may be influenced by large spatial factors as well as local factors, depending on the dispersal ability of the organisms (Gonçalves-Souza et al. 2014; Mykra et al. 2007). For example, Viana et al. (2015) in a study on the distribution and diversity of aquatic plants and cladocerans across different lakes in Europe found that environmental variation could explain high species turnover at regional scales, suggesting an important role of local processes in determining the dynamics of these communities.

In this context, our sampling strategy encompassing three hierarchical levels (different bromeliad species, different individuals per species, different tanks per individual) allowed interesting insights into the complexity of these ecological systems. We observed remarkable variation at all three levels (see Fig. 1, 2, 4, 5). There were species-specific patterns of alpha diversity (Fig. 3), beta diversity (Fig. 4 and 5), and community composition (Fig. 1 and 2), including taxa whose abundance was significantly different among host plants (Tables 2 and 3). There were also consistent differences in community composition among individuals of the same species, especially for eukaryotes, and more pronouncedly in *V. friburgensis* than in the other bromeliads (see Fig. 4 and 5). An intriguing observation was the striking difference in beta-diversity patterns between *V. friburgensis* and *V. platynema*: The former exhibited the highest intersample variability in community composition (e.g. see unweighted UniFrac results in Fig. 4 and 5), although its individuals were all sampled from very close locations in the same area (see Fig. S1). In contrast, the latter was the species whose samples were the most broadly spaced (see Fig. S1), but its phytotelm communities were much more similar to each other, for both prokaryotes and eukaryotes (Fig. 4 and 5). These findings suggest that biological differences between bromeliad species belonging to the same genus influence the degree of heterogeneity among their phytotelm communities.

The third hierarchical level (variation among tanks from the same bromeliad) revealed the most surprising results, as we observed substantial differences among such communities (Fig. 1, 2, 4, 5). Remarkably, in some cases samples from distinct bromeliad species were more similar to each other than pairs of samples from the same bromeliad individual (see Fig. 4 and 5). For example, the prokaryotic communities in the cisterns of some *A. gamosepala* individuals were more similar to those found in *V. platynema* samples (e.g. compare AGA2L2 and VPL4L2) than to other samples from the same bromeliad. This similarity, in some cases, might be explained by the spatial location of the host plants, such as AGA2 and VPL1 (Fig. S1). These plants are located in the same sampled patch, which could facilitate the exchange of organisms through dispersion. However, in other cases (e.g. AGA2 and VPL4), plants that are located in different patches showed a similar prokaryotic assemblage, indicating that factors other than dispersion may be involved in the shaping of these communities.

Such variation among tanks from the same bromeliad was also observed for eukaryotes, although here there was a clearer trend for samples from each host plant to cluster together (see Fig. 5). Still, we did observe cases in which interspecies differences (e.g. AGA4L1 vs. VPL2C) could be smaller than intraindividual differences (e.g. AG4L1 vs. AG4C). Interestingly, the similarity among cisterns from different host plants became more evident in the weighted UniFrac analyses, which takes into account the abundance of taxa and not only their presence or absence. Such cases of striking similarity between microbial communities harbored by different bromeliads suggest that an exchange of organisms among plant individuals of different species is not unusual. Overall, we can conclude that, in addition to dispersion, stochastic events such as wind-borne particles, fecal pellets, and liquid excretions of terrestrial animals, as well as dead leaves and animals, along with local environmental factors, can drive the community composition of such complex systems. This might help explain why some nearby samples (including different tanks from the same individual) can be more different

from each other than distant samples or samples from different bromeliad species.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Location of sample collection sites at the Pro-Mata Research Center, in the Atlantic Forest of southern Brazil.