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# Aquatic Microbial Habitats Within a Neotropical Rainforest: Bromeliads and pH-Associated Trends in Bacterial Diversity and Composition

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**Abstract** Tank-forming bromeliads, suspended in the rainforest canopy, possess foliage arranged in compact rosettes capable of long-term retention of rainwater. This large and unique aquatic habitat is inhabited by microorganisms involved in the important decomposition of impounded material. Moreover, these communities are likely influenced by environmental factors such as pH, oxygen, and light. Bacterial community composition and diversity was determined for the tanks of several bromeliad species (*Aechmea* and *Werauhia*) from northern Costa Rica, which span a range of parameters, including tank morphology and pH. These were compared with a nearby forest soil sample, an artificial tank (amber bottle), and a commercially available species (*Aechmea*). Bacterial community diversity, as measured by 16S rRNA analysis and tRFLP, showed a significant positive correlation with tank pH. A majority of 16S rRNA bacterial phylotypes found in association with acidic bromeliad tanks of pH<5.1 were affiliated with the *Alphaproteobacteria*, *Acidobacteria*, *Planctomycetes*, and *Bacteroidetes*, and were similar to those found in acidic peat bogs, yet distinct from the underlying soil community. In contrast, bromeliads with tank pH>5.3, including the commercial bromeliad with the highest pH (6.7), were dominated by *Betaproteobacteria*, *Firmicutes*, and *Bacteroidetes*. To empirically determine the effect of pH on bacterial community, the tank pH of a specimen of *Aechmea* was depressed, in the field, from 6.5 to 4.5, for 62 days. The resulting community changed predictably with decreased abundance of *Betaproteobacteria* and *Firmicutes* and a concomitant increase in *Alphaproteobacteria* and *Acidobacteria*. Collectively, these results suggest that bromeliad tanks provide important habitats for a diverse microbial community, distinct from the surrounding environment, which are influenced greatly by acid–base conditions. Additionally, total organic carbon (~46%) and nitrogen (~2%) of bromeliad-impounded sediment was elevated relative to soil and gene surveys confirmed the presence of both chitinases and nitrogenases, suggesting that bromeliad tanks may provide important habitats for microbes involved in the biological cycling of carbon and nitrogen in tropical forests.

## Introduction

Plants within the family *Bromeliaceae* are known for their capacity for extreme epiphytism, sometimes growing on bare rock or suspended from vines. Tank-forming bromeliads, in particular, are those that possess foliage arranged in a compact rosette capable of retaining water (otherwise known as phytotelmata; Fig. 1). Tank-forming bromeliads are known to dramatically influence the local macroecology of both aquatic and terrestrial organisms, due to tank use and overlapping leaves as shelter [4, 15, 28, 35]. In lieu of uptake via root systems, these plants are thought to rely on the tanks and products of decomposition of impounded material (litter and animals) for water and nutrients, respectively [3, 44].

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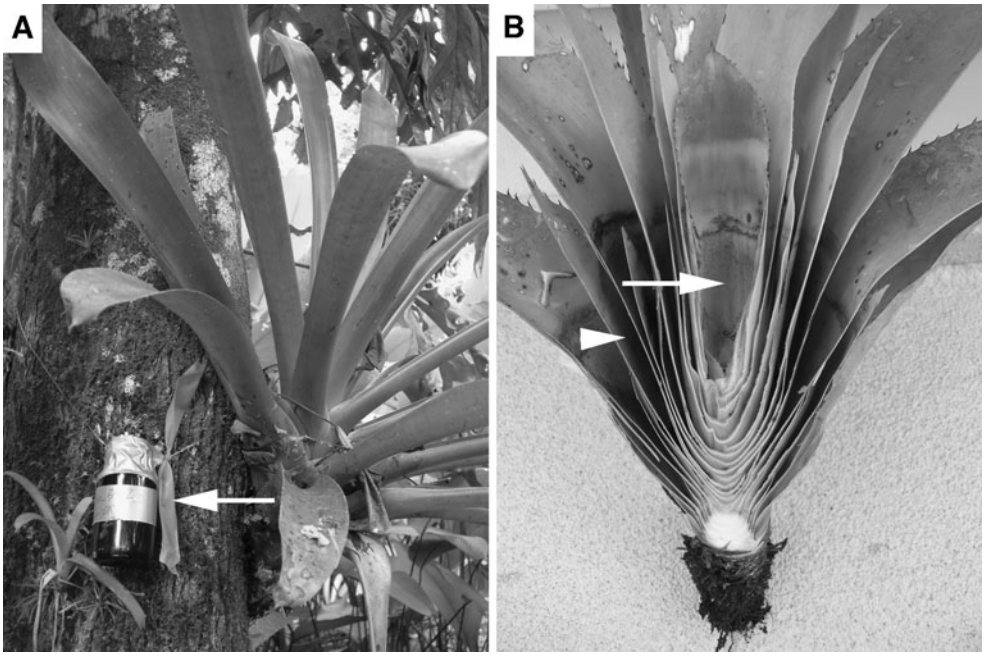


**Figure 1** Bromeliad species used in this study. **a** *Werauhia gladioliflora* ('*Wg*'), with nearby amber bottle (*arrow*) intended to artificially simulate a bromeliad tank. **b** Cut-away of *Aechmea nudicaulis*. Each bromeliad possesses a central rosette comprised of trough-like leaves that focus precipitation and runoff into the tanks. Younger leaves are deeper in the central tank (*arrow*) and mature leaves are in a series of closely spaced, shallower lateral tanks (*arrowhead*)

The suspended water within bromeliad tanks provides a unique niche in that water-saturated, acidic and anaerobic conditions do not typically occur in other locations within the canopy of tropical forests. Pittl et al. [34] investigated "arboreal soils", including the debris within bromeliad tanks in a lowland rainforest in Costa Rica, and found them to differ, as compared with ground soils, with much higher percent organic content, total carbon, and nitrogen, and smaller moisture and temperature fluctuations. The "micro-limnology" of these impoundments has been the focus of many studies, including measures of physical-chemical parameters, pH 4–6, $O_2$ levels as low as 0.5 ppm, and variable salinity [17], as well as calculations of their numerical relevance within rainforests [6, 33]. Bromeliad average density, for example, has been estimated at 1,000s to 100,000s $ha^{-1}$ ground area, depending on the study, with abundance as high as 175,000 mature plants $ha^{-1}$ estimated for a Columbian cloud forest [35, 39]. Fish [15] estimated that these densities within the cloud forest, may represent as much as 50,000 L suspended water in the canopy $ha^{-1}$, a value that has potential significance with regard to global biogeochemical cycling, including $CO_2$ and $CH_4$ efflux and organic carbon storage.

Despite their potential importance within neotropical habitats, little is known about the microbial communities within bromeliad tanks. At present, there are only a handful of studies investigating microbial diversity and processes within these unusual tropical habitats. For example, bacterial abundance was shown to be higher in bromeliad tank soils than ground soils, with a community dominated by the *Pseudomonadaceae*, *Bacillaceae*, and *Micro-coccaceae* and suggested to depend on plant location and stage of carbon decomposition [34]. *Bacillaceae* were similarly enriched from the leaf surface of a subfamily of bromeliads, *Till-andsioideae*, and found to be capable of nitrogen fixation [8]. Inselsbacher et al. [21] provided indirect evidence for a diverse and active bromeliad-associated microbial community, in the Atlantic Forest of Brazil, involved specifically in ammonification. Finally, Guimaraes-Souza et al. [17] measured bacterial respiration to be frequently higher than production, indicating a generally heterotrophic community within the tanks of bromeliads.

Bromeliads collectively suspend large amounts of water in the canopy, and thus represent a large environmental resource within the rainforest. As a group, they vary greatly in shape, size, host tree preference, and therefore litter fall composition and tank pH. It is likely that microbial diversity within bromeliad tanks may be influenced by any number of these variables, and given the important role of bromeliads in the nutritional and ecological status of the local habitat, it is not only necessary to understand the microbial composition within this unique habitat but also the possible sources of variation. Due to the small size and defined limits of bromeliad phytotelmata, they allow for the investigation of whole ecosystem complexity with the possibility of replication. The purpose of this study was to characterize the bacterial community within three species of tank-forming bromeliads in and around the La Selva Biological Station in northern Costa Rica, with regard to taxonomic identity and function, and to determine the possible influence of host plant species, morphology or tank pH on overall community structure.

## Materials and Methods

### Sample Collection

La Selva Biological Station, situated in a wet (4 m annual rainfall) lowland neotropical forest in northern Costa Rica, is located at the confluence of the Sarapiqui and Puerto Viejo rivers in the province of Heredia, Costa Rica (10°26′ N, 83°59′ W). The reserve, which covers approximately 1,600 ha, is home to dozens of bromeliads species, for which microbial samples from three, including those within the genera *Werauhia* (syn. *Vriesea*) and *Aechmea,* were collected in June 2009 and June 2010 (Fig. 1; Electronic Supplementary Material (Fig. S1); Table 1). *Werauhia* and *Aechmea* encompass a range of tank morphologies and pH conditions. This study included tank water from five specimens of *Aechmea mariae-reginae* ("*Amr*"), five specimens of *Aechmea nudicaulis* ("*An*", including one specimen, *An*47, from ~30 m in the canopy), nine specimens of *Werauhia gladioliflora* ("*Wg*"), including a paired soil sample collected from near and below *Wg*29, one specimen of *Aechmea fasciata*, a commercially available bromeliad species acquired locally and maintained for several months in a greenhouse at Occidental College, and an amber bottle (~100 ml vol.) intended to artificially simulate a bromeliad tank, attached to *Amr*1 for a duration of 12 mos (Fig. 1a). For one bromeliad (*Amr*1), the pH was artificially depressed, from 6.5 to ~4.5 for a total of 62 days, by the frequent addition of one to three drops of 1N-hydrochloric acid to the tank (June 2010). Unless otherwise noted, all bromelidas were within 3 m height on a host tree, usually within forest clearings, either mad-made or natural. Tank water samples were collected via serological pipette and transported to the lab in clean 15-ml plastic tubes.

### Chemical Analyses

Tank pH was measured via hand-held pH electrode (Hanna Instruments HI-98103B) in the field, prior to sampling for DNA analysis. For percent carbon and nitrogen, as well as stable isotopes on bulk sediments (Table 2), samples were dried at 65°C for 24 h, then milled to a fine powder. Dry material (0.5–2.0 mg) was placed into a tin capsule and combusted in a Eurovector (Milan, Italy) elemental analyzer. The resulting $N_2$ and $CO_2$ gases were separated by gas chromatography and admitted into the inlet of a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer for determination of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios. Typical precision of analyses was±0.5‰ for $\delta^{15}N$ and ±0.2‰ for $\delta^{13}C$ where $\delta=1{,}000\times(R_{sample}/R_{standard})-1$‰, where $R=^{15}N/^{14}N$, the standard for $\delta^{15}N$ is atmospheric nitrogen and Peedee Belemnite for $\delta^{13}C$.

### Phylogenetic Analyses

DNA extractions were performed, on station, within ~2 h of sample collection. Freshly collected bromeliad tank water samples (0.5 ml, including debris) were spun at 15,000×*g* for 10 min and the resulting pellet was extracted for total nucleic acids using the Power Soil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA). The protocol was modified by two initial 5–10-min incubations at 65°C followed by 5–10-min vortexing. The remainder of the extraction procedure was carried out according to the manufacturer's instructions, with the exception of a 4°C incubation in IRS solution (5 min) between solutions S2 and S3 to increase DNA yield and inhibitor removal. For 200–400-mg tank debris, DNA yield was approximately 6–40 ng $\mu l^{-1}$. SSU rRNA was amplified by polymerase chain reaction (PCR) from extracted DNA, according to [16, 26]. PCR products were pooled and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clone libraries of PCR amplified bacterial 16S rRNA genes were constructed from each bromeliad, with 23–79 clones analyzed for each library (Table 3). Transformants were screened directly for the presence of inserts using M13F/R vector primers (8-min initial denaturation). M13 amplicons were cleaned prior to sequencing with MultiScreen HTS plates (Millipore Corporation, Bedford, MA). Sequencing reactions were performed using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA), precipitated according to the manufacturer's instructions, and run on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Representative ribotypes (based on 97% sequence similarity) were selected for nearly full-length sequencing, using M13F/R primers, and 519F/1391R 16S rRNA gene internal primers.

Sequences were assembled and edited using Sequencher v4.2 (GeneCodes Corp.). Sequence homology searches were performed using BLASTn (NCBI), the Ribosomal Database Project classifier, and Greengenes [13, 42]. Additional sequences were obtained from GenBank and Greengenes and compiled and aligned, via the SILVA Aligner function, with our 16S rRNA sequences using the ARB Fast Aligner automated alignment tool with subsequent manual refinements [30]. Greengenes was also used to check for potential chimeras [13]. For near full-length representatives and closest relatives, neighbor-joining (NJ) analysis was conducted with Felsenstein distance correction. In some cases, partial sequences (~600 bp; $n$=47) recovered in our study were added to neighbor-joining trees in ARB via parsimony insertion within a tree of longer sequences (800–1,400 bp; $n$=101 sequences generated during this project). Maximum parsimony analysis was performed using the heuristic search option with 100 bootstrap replicates to assign confidence levels to nodes, shown in Figs. 6, 7, S2, S4, if >70% confidence

**Table 1** Bromeliads used in this study, including plant and tank morphometrics, tank pH, and location within the La Selva Biological Station, Costa Rica

| Bromeliad species | ID # | Plant | | | Tank | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Location[a] | Height (cm) | Width (cm) | pH | Height (cm) | Diameter (mm) | Library | tRFLP |
| *Werauhia gladioliflora* | 4 | STR 800 | 67 | 115 | 4.45 | 23 | 19 | | + |
| | 6 | STR 800 | 40 | 75 | 5.5 | 16 | 21 | | + |
| | 9 | SOR 450 | 34 | 48 | 4.7 | 8 | 22 | | + |
| | 10 | SOR 450 | 36 | 57 | 4.15 | 15 | 21 | | + |
| | 25 | SURA 625 | 78 | 167 | 4.08 | 25 | 30 | | + |
| | 29 | SURA 625 | 49 | 115 | 5.34 | 21 | 25 | | + |
| | 31 | SURA 625 | 48 | 150 | 5.90 | 19 | 23 | | + |
| | 37 | Lab clearing | 55 | 107 | 4.8 | 18 | 30 | + | + |
| | 39 | SOR 450 | 11 | 16 | 3.96 | 6 | 10 | | + |
| *Aechmea nudicaulis* | 22 | CCL 350 | 26 | 24 | 5 | 11 | 18 | | + |
| | 42 | CCL 350 | 25 | 30 | 5.1 | 8 | 19 | | + |
| | 44 | CCL 350 | 17 | 15 | 4.5 | 7 | 16 | | + |
| | 45 | CCL 350 | 19 | 20 | 4.7 | 8 | 18 | + | + |
| | 47 | CES 550 | 20 | 20 | nm | nm | nm | + | |
| *Aechmea mariae-reginae* | 1 | STR 770 | 114 | 145 | 5.8 | 44 | nm | + | + |
| | 2 | STR 770 | 46 | 57 | 6.3 | 16 | 18 | + | + |
| | 12 | SOR 450 | 64 | 151 | 5.63 | 15 | 15 | | + |
| | 34 | SCH 100 | 30 | 62 | 5.45 | 9 | 17 | | + |
| | 50 | Lab clearing | 77 | 132 | 5.6 | 24 | nm | + | + |
| *Aechmea fasciata* | – | Commercial | nm | nm | 6.7 | nm | nm | + | |
| Artificial tank[b] | 1 | STR 770 | – | – | 6.1 | – | – | + | |
| Soil[c] | 31 | SURA 625 | – | – | – | – | – | + | |

*STR* Sendero Tres Rios, *SOR* Sendero Oriental, *SURA* Sendero Surá, *CCL* Camino Circular Lejano, *CES* Camino Experimental Sur, *SCH* Sendero La Chanchera, (+) designates which samples yielded results acquired via either clone library construction or tRFLP

[a] Trails at La Selva are signed 50 m each indicating the acronym of the trail and the distance, in meters, from the lab clearing

[b] An amber bottle (~100 ml vol.) intended to artificially simulate a bromeliad tank, attached to *Amr*1 for a duration of 12 months

[c] Collected from near and below *Wg*29

**Table 2** Summary of average total carbon and nitrogen content, as well as stable isotopic composition, for bromeliad, and soil, samples investigated in this study (standard deviation in parentheses)

| Bromeliad species[a] | Plant | | | | Tank[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | C (%) | $\delta^{13}$C (‰) | N (%) | $\delta^{15}$N (‰) | C (%) | $\delta^{13}$C (‰) | N (%) | $\delta^{15}$N (‰) |
| *Werauhia gladioliflora* | 48.2 (7.1) | −29.3 (0.3) | 0.4 (0.1) | −2.2 (0.2) | 45.8 (2.0) | −29.8 (0.4) | 2.0 (0.3) | −0.3 (1.4) |
| *Werauhia kupperiana* | 42.1 (0.9) | −29.2 (0.6) | 0.2 (0.0) | −0.5 (0.4) | 44.5 (3.9) | −29.7 (0.4) | 2.4 (0.1) | 2.2 (1.1) |
| *Aechmea nudicaulis* | 40.5 (5.4) | −16.0 (1.4) | 0.3 (0.2) | −1.9 (0.9) | 47.2 (3.1) | −29.7 (0.3) | 1.9 (0.4) | 1.1 (1.6) |
| *Aechmea mariae-reginae* | 42.6 (2.1) | −14.1 (0.7) | 0.3 (0.1) | −0.4 (0.2) | 43.2 (0.7) | −28.4 (1.4) | 1.9 (0.6) | 2.1 (1.2) |
| Soil[c] | na | | | | 6.5 (3.8) | −27.8 (1.1) | 0.3 (0.1) | 4.2 (0.2) |

[a] $n$=2 for *W. gladiolifora, W. kupperiana*, and *A. mariae-reginae*; $n$=4 for *A. nudicaulis*; $n$=3 for soil

[b] Except for soil

[c] Collected from near and below *Wg*29

**Table 3** Summary of bacterial ribosomal 16S rRNA clone library results

| Phylogenetic group[a] | Soil31 | *An*45 | *An*47 | *Wg*37 | *Amr*2 | *Amr*1 | *Amr*50 | *Af* | Btl1[b] |
|---|---|---|---|---|---|---|---|---|---|
| Acidobacteria | | | | | | | | | |
| Subdivision 1 | 20 | 7 | 5 | 8 | | 3 | 1 | | 1 |
| Subdivision 3 | 10 | 9 | | 2 | 2 | 3 | 1 | | 1 |
| Subdivision 6 | 1 | | | | 1 | 2 | | | |
| Subdivision 8 | 2 | | | 5 | 6 | 1 | | | |
| Other | 7 | | | | 1 | 1 | | | 1 |
| Alphaproteobacteria | | | | | | | | | |
| Rhizobiales | 5 | 13 | 4 | 3 | 1 | 2 | 5 | 2 | 2 |
| Rhodobacterales | | | | 2 | | | | 2 | |
| Rhodospirillales | 5 | 5 | | | | | | | |
| Other | 1 | | | 1 | | 1 | | 1 | 1 |
| Betaproteobacteria | | | | | | | | | |
| Burkholderiales | 4 | 4 | 1 | 2 | 7 | 2 | 2 | 4 | 5 |
| Rhodocyclales | | | | 1 | 1 | 6 | 3 | 3 | 1 |
| Other | 1 | | 1 | 1 | | | | 6 | |
| Bacteroidetes | | | | | | | | | |
| Bacteroidales | | 3 | 5 | 1 | 6 | 6 | | | |
| Sphingobacteriales | | 2 | 1 | 1 | 7 | 3 | 2 | | 10 |
| Other | | 1 | | 2 | 4 | 2 | 1 | | 1 |
| Deltaproteobacteria | | | | | | | | | |
| Desulfobacterales | 1 | | | | 1 | 2 | | | |
| Desulfuromonadales | | 2 | | 2 | 2 | 2 | | | 1 |
| Myxococcales | 3 | 1 | | 1 | | 2 | | 1 | 2 |
| Syntrophobacterales | | 1 | 2 | 2 | 4 | 1 | | | |
| Gammaproteobacteria | | | | | | | | | |
| Methylococcales | | | | | 5 | 2 | | | |
| Xanthomonadales | 2 | 2 | | 1 | 1 | | | | 2 |
| Other | 3 | | | | 2 | 1 | | | 2 |
| Firmicutes | | | | | | | | | |
| Clostridiales | | 2 | | 5 | 2 | 4 | 5 | 7 | 4 |
| Other | | | 1 | | 1 | 4 | 1 | | |
| Verrucomicrobia | | | | | | | | | |
| Subdivision 3 | 8 | 1 | | 3 | | 4 | | | 3 |
| Other | 2 | 1 | 3 | 1 | 2 | 2 | | | 9 |
| Planctomycetes | 2 | 10 | 1 | 4 | 1 | | | 1 | 3 |
| Cyanobacteria | | 2 | | 2 | 12 | | | | |
| Other | 4 | | 3 | 5 | 5 | 11 | | 2 | 2 |
| Total | 79 | 66 | 27 | 55 | 78 | 62 | 23 | 27 | 52 |

*An Aechmea nudicaulis*, *Amr A. mariae-reginae*, *Af A. fasciata*, a commercially available bromeliad, *Wg Werauhia gladioliflora*. The soil sample collected from near and below *Wg*29

[a] The phylum Proteobacteria was further divided into classes; Alpha-, Beta-, Delta-, and Gammaproteobacteria

[b] Btl 1=an amber bottle (~100 ml vol) intended to artificially simulate a bromeliad tank, attached to *Amr*1 for a duration of 12 mos.

[PAUP*4.0b10; 40]. Ribotypes recovered from bromeliad tanks, and soil, spanned a wide phylogenetic range within each phylum and are shown in Figs. 6 and 7 and the Electronic Supplementary Material (Figs. S2 and S4).

## Terminal Restriction Fragment Length Polymorphism and Diversity

T-RFLP was used to characterize the relative proportions of coarse bacterial groups, and corresponding diversity associated with bromeliad samples. 16S rRNA genes from purified DNA samples, as described above, were PCR-amplified using bacterial primers 27F (fluorescently labeled with WellRED dye D3, Sigma-Proligo, St. Louis, MO) and 1492R, using the conditions described above for unlabelled PCR reactions. For a few samples that were difficult to amplify, the Illustra PureTaq Ready-To-Go™ PCR beads (GE Healthcare Life Sciences) were used, with the same protocol as above, with the exception of an anneal temperature of 56°C, to ensure the amplification of specific products. For each sample, duplicate

PCR amplifications were performed and pooled prior to digestion with *Rsa*I (for 6–8 h at 37°C, New England Biolabs, Beverly MA). Fluorescently labeled fragments were separated by capillary electrophoresis and analyzed on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Fragment sizes were parsed by separation of >3 bp, and relative abundances were estimated using the CEQ 8800 Fragment Analysis software. Diversity was assessed by the number of peaks obtained after restriction with *Rsa*I and assessed by the Shannon–Wiener index ($H'$) according to the equation $H'=-\sum(p_i)(\ln p_i)$, where $p_i$ was the normalized area under each T-RFLP fragment peak within each sample. For a comparison of bacterial community structure between different bromeliad samples, cluster analysis of the normalized T-RFLP data set was performed using PC-ORD 5.10 software (Euclidean distance measure, Ward's method for group linkage; 31]). Beals smoothing [1] was performed on the data matrix in order to correct for the large number of absent taxa in the data set and to assign probabilities to taxa occurrence within a sample unit based on occurrences within the full matrix. Additionally, an ordination was performed on the smoothed data by non-metric multidimensional scaling (NMS; Euclidian distance). A two-dimensional result was chosen based on comparison with 250 runs on randomized data for ordinations using one to six axes. The final stress value of the ordination was 4.857 and the likelihood of a similar value being derived from randomized data was 0.0040 (Monte Carlo simulation). Statistical correlations among data sets were analyzed via JMPv8.0.2 (SAS Institute, Inc.).

### Chitinase and Nitrogenase Gene Assays

PCR assays for both chitinase and nitrogenase were carried out for DNA tank water extracts of bromeliad *An*45 and *An*22, respectively. Chitinase (*chiA*) PCR mixtures contained ChiAF2 and ChiAR2 primers, which amplify *chiA* from a wide taxonomic range of bacteria from aquatic environments [45]. Genes that encode for the iron protein (*nifH*) within nitrogenase, the enzyme complex that catalyzes nitrogen fixation, were assessed using general primers, nifHf-10aa and nifHr-132aa, known to amplify nitrogenase genes from a variety of bacteria from aquatic environments [32]. Both thermal cycling protocols included 60 s each of denaturation at 94°C, annealing at 48.2°C and 50°C for *chiA* and *nifH*, respectively, elongation at 72°C (25–30 cycles), and a final 6 min of elongation at 72°C. Sequencing reactions were performed using the Genome Lab DTCS Quick Start Kit either directly, with PCR primers, or as clones with M13 primers, as described above for 16S rRNA genes. Nucleotide sequences were assembled and edited using Sequencher v4.2 (GeneCodes Corp.). Additional alignment and translations were performed using MacClade v4.0.8. Sequence homology searches within GenBank were performed using BLASTx (NCBI).

### Nucleotide Sequence Accession Numbers

Sequences obtained in this study have been deposited in the GenBank database under accession numbers HQ010132–HQ010279 (bacterial 16S rRNA ribotypes), HQ010126–HQ010131 (*chiA*), and HQ010120–HQ010125 (*nifH*).

## Results and Discussion

### Carbon and Nitrogen Dynamics

Bromeliads collect large amounts of organic material, dominated by plant litter and animals (plus remains), primarily insects and crustaceans. All bromeliad tanks showed high levels of total organic carbon (45.6±2.9% C; $n=10$), relative to soil conditions (6.5±3.8% C; $n=3$; Table 2). Tank debris $\delta^{13}C$ values were not significantly different among bromeliad species (−29.5±0.7‰; $n=10$) and, for the genera sampled in this study, plant tissue $\delta^{13}C$ values generally reflected carbon fixation pathway (~−15‰ for CAM and −29‰ for C3; Table 2). Solid debris ranged from 2 to 50 mg $ml^{-1}$ of tank fluid, with qualitative differences in consistency, ranging from flocculent to dense. The much greater total organic load in bromeliad tanks, as compared with soil, which was similarly observed by Pittl et al. [34], suggests a clearly different habitat with regard to heterotrophic potential, and it is likely that some bacteria present are capable of degrading pectin and cellulosic plant material, as well as chitin, the main component of arthropod exoskeletons. The longevity and stability of conditions deep in the tank make possible the effective turnover of these allochthonous sources of carbon. For example, chitin is an extremely resilient long chain polysaccharide and breakdown is usually catalyzed by chitinase enzymes, secreted by a variety of detritivorous bacteria [22]. Chitinase *chiA* genes, including those belonging to members of the *Firmicutes*, mostly *Bacillus* species, Proteobacterial subgroups, as well as some belonging to fungi, were recovered from the tank of *An*45 (Electronic Supplementary Material, Table S1). The presence of these genes suggests that the breakdown of recalcitrant chitin could play a significant role in the degradation of organic material in bromeliad tanks.

Total nitrogen in the tanks measured 2.0±0.4% N ($n=10$) and was comparably higher than both plant tissue and soil (both 0.2–0.4%; Table 2). $\delta^{15}N$ values were variable and ranged from −1.3‰ to 2.9‰ for tank material, −2.9‰ to −0.2‰ for plant tissue, and 4.2‰ for the soil sample (Table 2). This depletion in $\delta^{15}N$, relative to typical plant tissue, suggests either a reliance of the plant on rainfall or

fixed nitrogen, not sourced from soil. Active nitrogen fixation has been detected in epiphytic members of the bromeliad subfamily *Tillandsioideae* [8] and one report implicated cyanobacteria as the microbial member responsible for the activity [6]. Genes that encode for the presence of functional nitrogenase enzymes, belonging to both bacteria (*Clostridiales*, *Rhodospirillales*, and *Rhizobiales*) and archaea (*Methanomicrobiales*), were recovered from bromeliad *An*22 and were similar to those recovered previously from wood-feeding termites [46], a rice rhizosphere and a Panamanian bromeliad (Electronic Supplementary Material, Table S1). Tank bromeliads live in an environment that is low in bioavailable nitrogen and have no extensive root system for the uptake of ammonium, thus, nitrogen fixation by bacteria within the tanks, if occurring, could facilitate their epiphytic lifestyle.

## Diversity of Bromeliad-Associated Bacteria: Influence of Tank pH

A comparison was made of bacterial community structure among three species of bromeliad (*A. nudicaulis*, *A. mariae-reginae*, and *Werauhia gladioliflora*), encompassing a range of tank pH and morphologies. With regard to influence of bromeliad species specifically, Fig. 2 shows that despite distinct clustering of bacterial community similarity between the two species of *Aechmea*, the trend is complicated by the addition of *W. gladioliflora* data. Cluster analysis from all three bromeliad species suggested that pH was a stronger influence on bacterial community composition, with an average pH value for each cluster that was markedly different (4.0–5.1 versus 5.3–6.3; Fig. 2a). Some taxa were clearly responding to this pH threshold, with 12 T-RFLP fragments that appeared only when the pH was higher. NMS ordination, however, suggested that the community overall was responding in a continuous fashion to pH, rather than simply a threshold effect (Fig. 2b). The dominant axis pulled out by the ordination shows a correlation coefficient of ~0.8 for tank pH and no more than ~0.5 for any other variable measured, supporting the contention that pH is driving the variability in the dataset. Similarly, tank pH was the best predictor of bacterial community diversity, as measured by the Shannon–Wiener index calculated from T-RFLP data, (regression $P$=0.019; Fig. 3), compared with other parameters, including bromeliad species, tank size (height, diameter, and volume), and plant size (height and width; all $P$>0.07, with the exception of plant width, $P$=0.036, data not shown). The correlation between pH and microbial diversity has also been observed for other chemically extreme, organically rich environments [2, 7, 23, 48]. For example, the lowest bacterial diversity was found in temperate and tropical forest soils with pH of <4.5 and, conversely, increased diversity was observed for non-agricultural and wetland soils at higher, near-neutral pH [19, 27]. The commercial bromeliad in this study, with the highest tank pH measured (6.7) and a low diversity (only 13 phylogenetic families, as designated by RDP classifier, compared with 46–60 families for the wild bromeliads) was the exception, however, this is thought to represent a significant perturbation from normal community conditions (see also the Electronic Supplementary Materials).

## General Trends in Bromeliad Bacterial Community Composition: Influence of Tank pH

The bromeliad tank environment contained a diverse bacterial community. Eighty-five bacterial families, as assigned by the RDP classifier database, were identified within clone libraries, with the majority affiliated with six major phylum-level groups (including the Proteobacteria, which were divided further into four classes; Table 3). Bromeliad environment was separated into acidic (pH 4–5.1), less acidic (pH 5.3–6.3), and commercial (pH 6.7) conditions. The acidic and less-acidic natural bromeliads had 25 bacterial families in common, with 21 and 35 unique families between them, respectively. Molecular analysis of the bacterial community in acidic bromeliad tanks of pH 4–5.1 (bromeliad #s *Wg*37, *An*45, *An*47), revealed a dominance of *Acidobacteria* (average, 23±4% of recovered ribotypes) and *Alphaproteobacteria* (18±9%), an abundance of *Bacteroidetes* (13±8%) and *Planctomycetes* (9±6%), and to a lesser extent, *Verrucomicrobia*, *Betaproteobacteria*, and *Deltaproteobacteria* (~7%; Fig. 4, Table 3, see also the Electronic Supplementary Materials). Many of the recovered bacterial 16S rRNA ribotypes were related, in some cases, to microbes previously observed in soil, rice paddies, peat bog, and stagnant water [9, 12, 24, 36, 47]. Additionally, the overall community make-up, with regard to phylum/class level abundances, was strikingly similar to those found in acidic, water-logged, peat bog habitats that contained both a shallow oxic and well-developed anoxic layer, with significant levels of decomposition (Fig. 4; [12, 24]). A soil community sampled within the vicinity of bromeliad *Wg*37 was also comprised mainly of *Acidobacteria* (56% of recovered clones) and *Alphaproteobacteria* (14%; Fig. 4). For other less acidic bromeliads with tank pH of 5.3–6.3 (bromeliad #'s *Amr*1, *Amr* 2, and *Amr*50), however, the dominant bacterial groups included *Bacteroidetes* (18±4%) and, conversely, *Betaproteobacteria* (15±6%) and *Firmicutes* (14±11%), with similar representation of other less abundant groups, including *Verrucomicrobia* and *Deltaproteobacteria* (Fig. 4; Table 3). Many of these were also related (>95% 16S rRNA similarity) to those recovered from river sediment, activated sludge, rhizosphere, and wetlands (Figs. 6 and 7; Electronic Supplementary Material, Figs. S2 and S4)). These trends revealed a general difference in dominant bacterial groups from *Acidobacteria* and *Alphaproteobacteria* at lower pH conditions to *Betaproteobacteria* and *Firmicutes* at higher pH levels within bromeliad tanks.

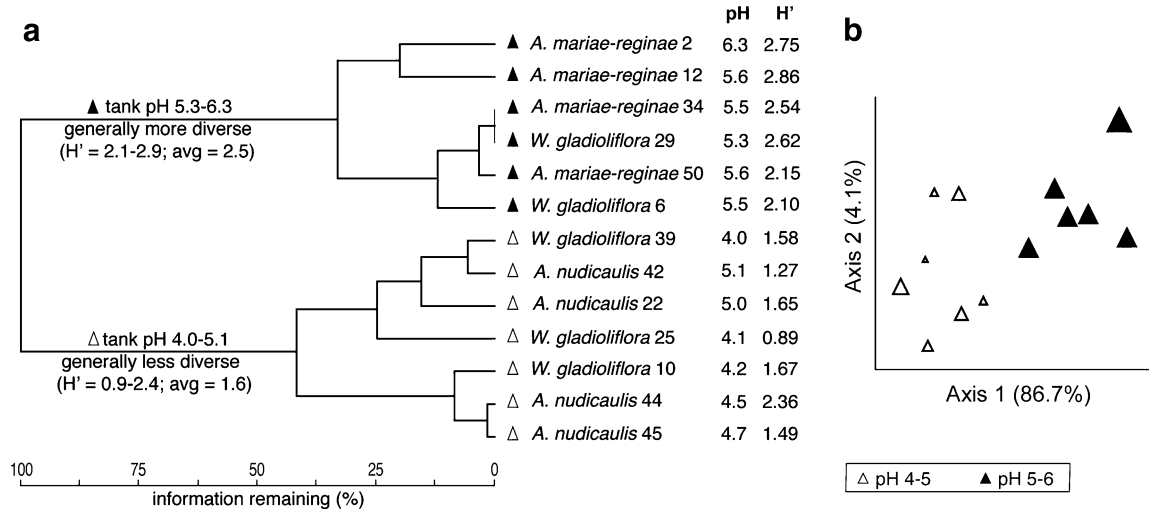


**Figure 2** Overall variance in bacterial community composition as analyzed by T-RFLP fingerprinting (both identity and relative abundance). **a** Cluster analysis of community composition for the tank water of 13 bromeliad individuals, comprising three species. The clades are distinguishable by tank pH and Shannon–Wiener diversity index (*H'*), both listed after the name of each bromeliad, in that order. **b** NMS ordination of community composition for each bromeliad, emphasizing the distinct delineation in pH-resolved community structure (*empty triangles*, pH 4.0–5.1; *filled triangles*, pH 5.3–6.3). Axis 1 exhibits a strong correlation with tank pH ($r$=0.794)

To empirically determine the effect of pH on bacterial community composition, the tank pH of a specimen of *A. mariae-reginae* (*Amr*1) was depressed (from 6.5 to 4.5) for 62 days. The resulting community changed predictably with decreased abundance of *Betaproteobacteria* (from 12.9% to 4.1%) and *Firmicutes* (from 12.9% to 2.0%) with a concomitant increase in *Alphaproteobacteria* (from 4.8% to 8.2%) and *Acidobacteria* (from 16.1% to 30.6%; Fig. 5). In previous studies, differences in soil pH resulted in comparable differences in bacterial composition for non-agricultural soils as well [19, 27].

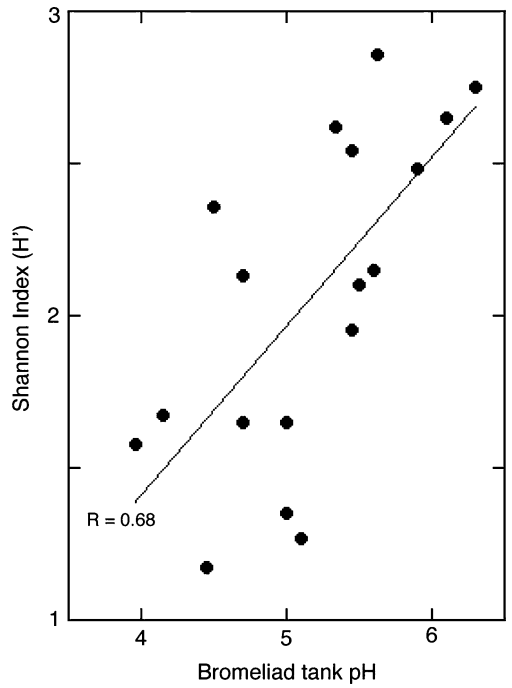


**Figure 3** A comparison of the Shannon-Wiener diversity index for each bromeliad, as determined by tRFLP, versus tank pH, suggesting that bacterial populations were less diverse at lower tank pH's ($n$=18; $y$=−0.81+0.55$x$; $R$=0.68; regression, $P$=0.019). Only those bromeliads with a '+' in the tRFLP column in Table 1 were included

## Phylogenetic Identity of pH-Dependent Bacterial Groups

Many of the bacterial 16S rRNA ribotypes recovered had presumed phenotypes that were congruent with characteristics of the tank environment (acidophilic and facultatively anaerobic). For example, *Acidobacteria* were dominant in acidic bromeliad tank water and widely distributed among subdivisions (Table 3; Fig. 6). In bromeliad tanks, *Acidobacteria* comprised up to 27% of the recovered diversity, but associated ribotypes generally associated with only three groups (subdivisions 1, 3, and 8; Table 3; Fig. 6). *Acidobacteria* in this study comprised over 50% of the library for the soil sample, with members of at least seven clades/subclades represented (Fig. 6), suggesting that the bromeliad ecosystem is unique from the surrounding soil. *Acidobacteria* are often the most abundant bacteria represented in molecular surveys of soil environments and the relative abundance of subdivision 1 *Acidobacteria*, in particular, is known to vary with pH [14, 37]. This was the case for bromeliad communities as well, where subdivision 1 *Acidobacteria* comprised 10–18% of the recovered ribotypes from acidic tanks (pH 4–5.1), and only 0–5% of tanks with higher pH (5.3–6.3). *Acidobacteria* thrive primarily in acidic environments and genomic analysis of isolates has revealed diverse heterotrophic metabolism, chitin and cellulose utilization, and desiccation resistance [43], suggesting that

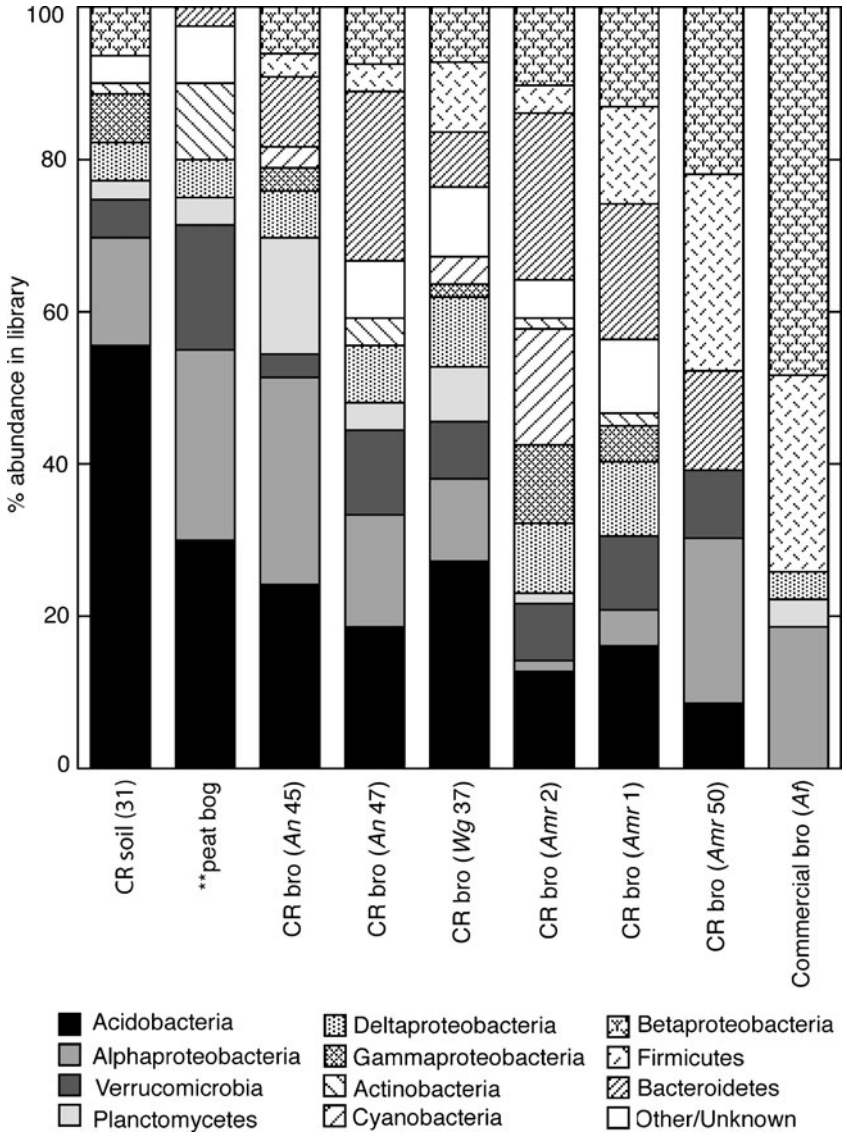


**Figure 4** Differences in the abundance of bacterial subgroups recovered from Costa Rican bromeliad tank water, versus those recovered from underlying soil, a commercially available bromeliad, and from peat bog (*double asterisks*, results taken from Dedysh et al. 2006 [12]). The phylum Proteobacteria was further divided into classes; Alpha-, Beta-, Delta-, and Gammaproteobacteria. *An Aechmea nudicaulis*, *Amr Aechmea mariae-reginae*, *Af Aechmea fasciata*, a commercially available bromeliad, *Wg Werauhia gladioliflora*. The soil sample (soil31) was collected from near and below *Wg*29

they are well-suited for life within bromeliad tanks. Many, including the chemoorganotrophic *Holophagales* (subdivision 8), may be important in the breakdown of litter within the bromeliad tank environment [14].

*Alphaproteobacteria* were well represented (11–27%) within most tank bromeliads, with the exception of bromeliads *Amr*1 and *Amr*2 (1–5%; Table 3). Members of the order *Rhizobiales* were represented in all samples, although in greater abundance in more acidic tanks and soil (Table 3; Fig. 7). The recovered ribotypes were diverse and spread over six families, with many related to cultured members of the photoheterotrophic *Rhodoblastus*, the chemoorganotroph *Rhodomicrobium*, and the generally methanotrophic, acidophilic genera *Methylocapsa* and *Methylocystis*, all previously isolated from *Sphagnum* peat bogs and acidic tropical forest soil [10, 11, 18]. Additionally, *Alphaproteobacteria* within the *Rhodospirillales* and *Rhodobacterales* were present in tanks with pH<5.1. Many of these bacteria, including *Acidisphaera* and *Rhodobacter* (97–100% similarity to recovered clones), are known to have a preference for low pH and are commonly found in aquatic habitats with significant amounts of soluble organic matter, low oxygen tension, and sufficient light levels, such as wastewater catchments, coastal lagoons, and rice paddy fields [20].

*Betaproteobacteria*, which are widespread in soil environments, were present, and sometimes abundant, within the bromeliad tanks (6–22% for non-commercial bromeliads, 48% for the commercial sample), with relatives from at least three different families recovered, including the *Burkholderiales* recovered from all samples, regardless of type (bromeliad, soil, or artificial tank) or pH (Table 3, Fig. 7). Several groups appeared more abundant in less acidic bromeliads (*Amr*1, *Amr*2, *Amr*50), including members of the *Comamonadaceae* (*Burkholderiales*) and *Rhodocyclaceae* (*Rhodocyclales*). The *Rhodocyclaceae*, in

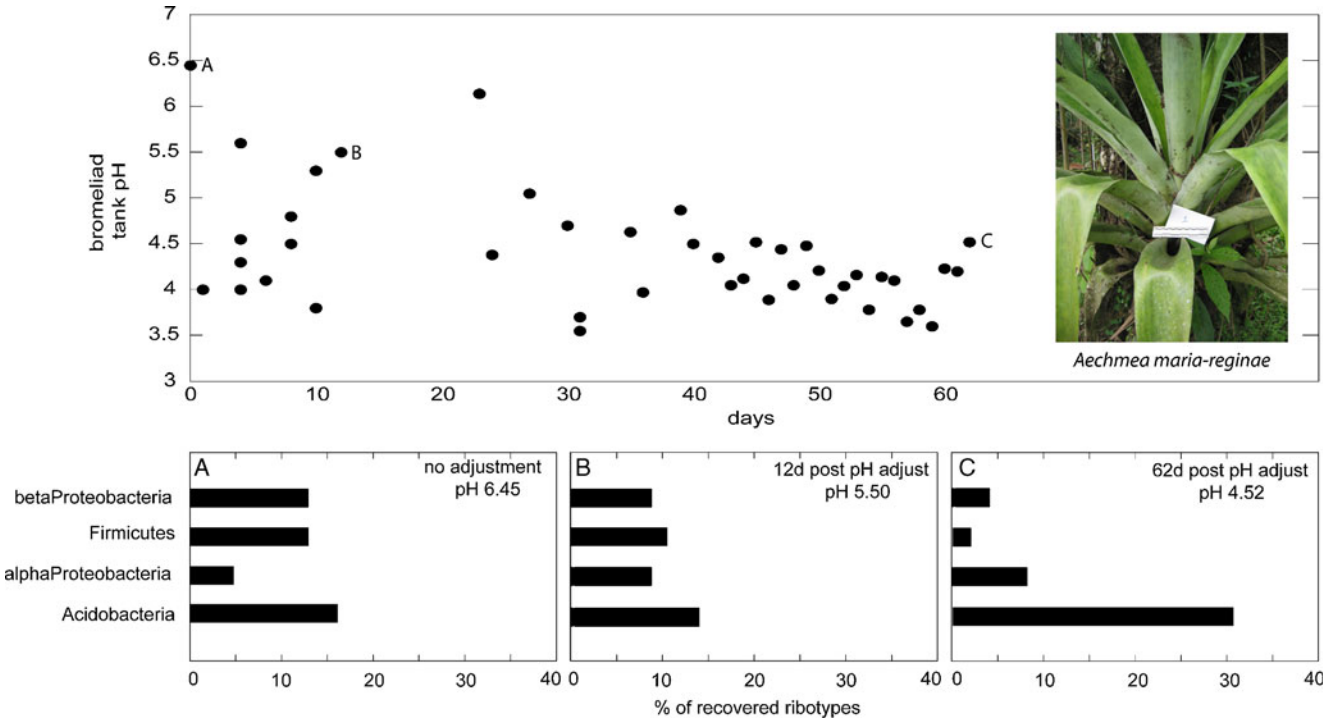


**Figure 5** Long-term pH manipulation of the tank water of a Costa Rican bromeliad (*Aechmea maria-reginae*) in situ, and the resulting changes in abundance (% of recovered ribotypes) of four bacterial subgroups, shown previously to vary depending on the natural acid–base conditions within the tank. The *top panel* shows the pH within the bromeliad tank over the course of the experiment from days 0 to 62. Time points include; *A* prior to manipulation (no pH adjustment, which also corresponds to *Amr*1 in Fig. 4) and 12 days (*B*) and 62 days (*C*) post-manipulation of the acid–base conditions, from the natural pH of 6.45 to the final target pH of 4.52. In the field, pH was adjusted initially and daily, when necessary, by the addition of 1N-hydrochloric acid. $n$=57, 57, and 49 clones analyzed for *A* (time 0), *B* (time 12 days), and *C* (time 62 days), respectively

particular, are known for their presence in activated sludge, soil, and anoxic freshwater sediments [29, 38, 41].

*Firmicutes* were the most variable group, in term of relative abundance within the bromeliad environment, ranging from 5% to 14% of recovered ribotypes in acidic versus less acidic tanks, respectively, and 26% in the commercially grown bromeliad (Table 3). Although members of this group are metabolically diverse and reported from a range of environments, ribotypes recovered in our study were overwhelmingly related to members recovered from sewage, anaerobic sludge, and the gut microbiome (Fig. S4). Similar to *Bacteroidetes*, *Firmicutes* were not recovered from the soil sample and did appear to preferentially inhabit bromeliad tanks (see the Electronic Supplementary Materials).

## Factors that Influence Bromeliad Tank pH

Acid–base conditions appear to be a major influence on bacterial community structure and diversity within tropical bromeliads, but the specific controls on pH conditions within the tank (e.g., primary fermenter community composition, host tree source leaves, amount of debris, etc.) is not known. Benzing et al. [5] found tank pH to be more than a simple function of dissolved $CO_2$, suggesting additional influence besides plant metabolism. High $CO_2$ (up to 47 ppm) and low $O_2$ (<8 ppm) are thought to be maintained by tank microbiota and not the plant itself [25], however, details regarding the specific role of microbes in pH mediation are lacking. We suspect the production of volatile fatty acids by fermentative bacteria, of which we recovered many. Additionally, Laessle [25] showed that tank pH, $O_2$, and $CO_2$ conditions were affected by exposure to sunlight, as well as the amount and type of impounded litter, which, when abundant, caused significant $O_2$ depletion and $CO_2$ accumulation. Guimaraes-Souza et al. [17] similarly observed that bromeliad tanks in full sun had a lower pH (4.6), compared with those in shade (5.6), which we also observed in the field (W.W., personal observation). Merwin et al. [33] showed that host tree species influenced bromeliad distribution and that soil type altered host tree leaf biochemistry. This may ultimately affect the tank water conditions since these leaves would likely be the main source of impounded plant material. In support of this assertion, preliminary experiments in the field suggest that certain leaf types (ex. *Dipteryx* and *Virola*) resulted in less acidic tank conditions compared with bromeliad tanks filled with *Hyeronima* leaves (Goffredi, G. North, and C. Wilch, unpublished observation).

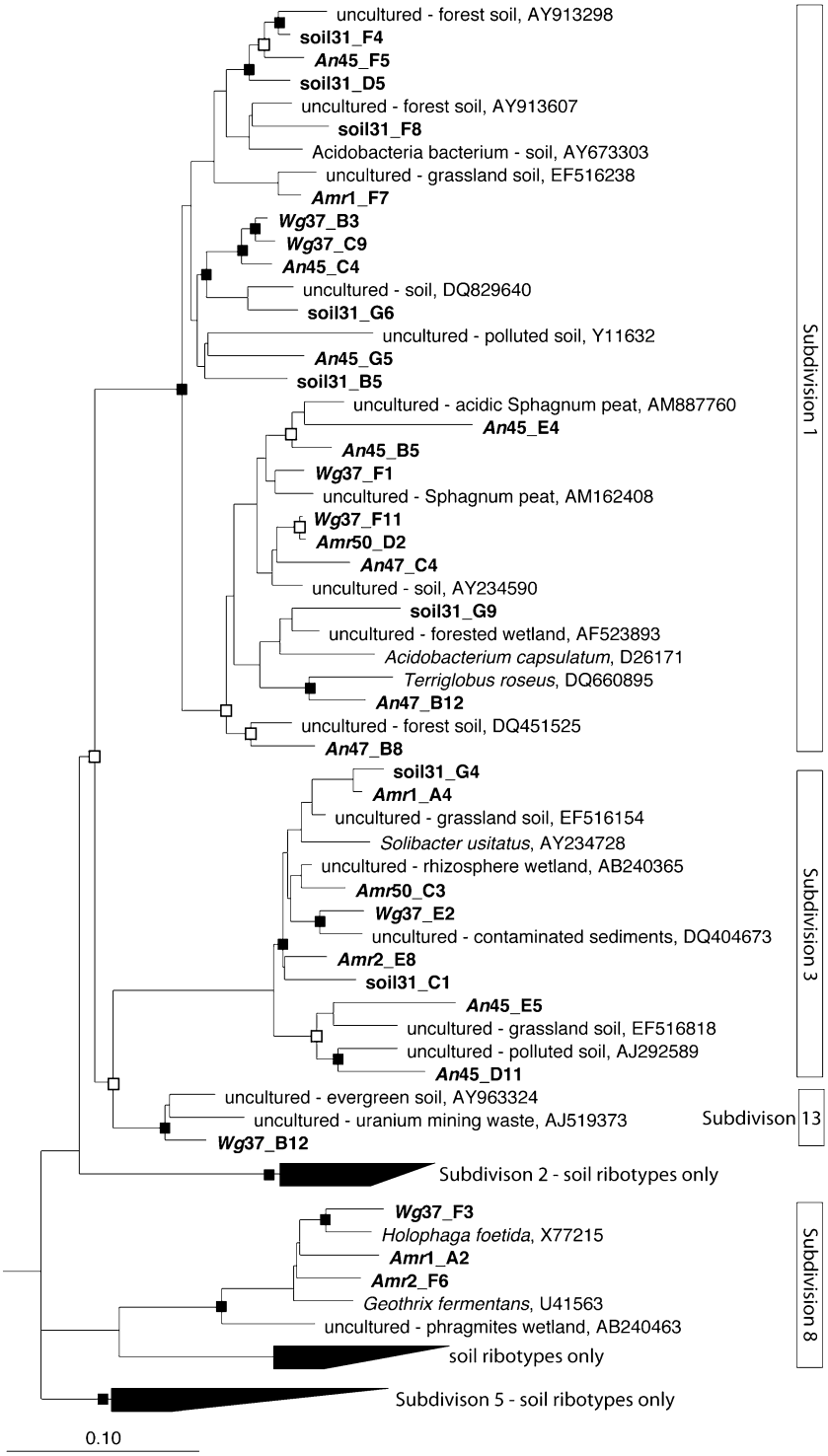


**Figure 6** Phylogenetic relationships among *Acidobacteria* associated with Costa Rican bromeliads and a nearby soil sample, relative to selected cultured and environmental sequences in public databases, based on sequence divergence within the 16S rRNA gene. A neighbor- joining tree with Kimura two-parameter distances is shown with *Rhodomicrobium vannielii* (AB250621) used as an outgroup (not shown). The symbols at the nodes represent bootstrap values from parsimony methods obtained from 100 replicate samplings (*empty squares*, 70–90% and *filled squares*, 90%+ bootstrap support)

## Conclusions

Microbial diversity and community structure within bromeliad tanks is different from the surrounding environment, and this diversity may ultimately contribute to the greater diversity of the rainforest. Both anaerobic and aerobic bacterial groups were present at high abundance, indicating the highly heterogeneous nature of this unique environment. Bacterial community diversity showed a significant correlation with tank pH and changes in pH influenced the resident microbial population with a general shift in dominant bacterial groups from *Betaproteobacteria* and *Firmicutes* at higher pH levels to *Acidobacteria* and *Alphaproteobacteria*, which were remarkably similar to

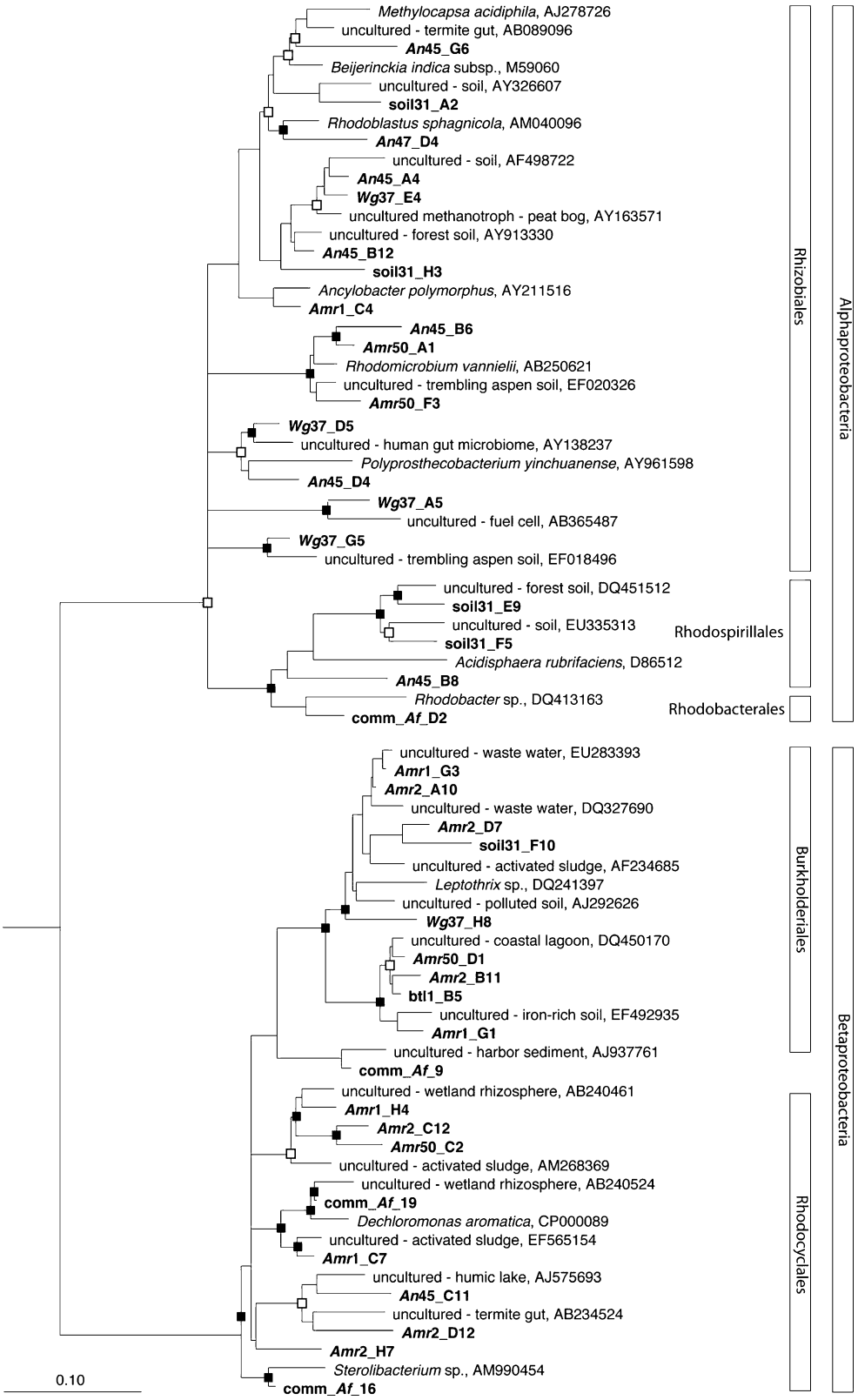


**Figure 7** Phylogenetic relationships among *Alpha*- and *Betaproteobacteria* associated with Costa Rican bromeliads, a commercially available bromeliad, an artificial tank, and a nearby soil sample, relative to selected cultured and environmental sequences in public databases, based on sequence divergence within the 16S rRNA gene. A neighbor-joining tree with Kimura two-parameter distances is shown with *Flavobacterium psychrophilum*, (AF090991) used as an outgroup (not shown). Some unsupported nodes were collapsed. The symbols at the nodes represent bootstrap values from parsimony methods obtained from 100 replicate samplings (*empty squares*, 70–90% and *filled squares*, 90%+ bootstrap support)

peat bog habitats, at lower pH conditions. Gene surveys confirmed the presence of chitinases (*chiA*) and nitrogenase genes (*nifH*), suggesting that bromeliad tanks may provide important habitats for microbes involved in the biological cycling of carbon and nitrogen in tropical forests. Small impoundments, such as bromeliads, are invaluable for investigating microbial biodiversity and the possible linkages between terrestrial and aquatic elemental cycling in tropical forests. Further analysis of carbon flow through the different compartments of the bromeliad microbiota is essential towards understanding the microbe-driven nutrient cycling in this unique microbial environment. Small impoundments, such as bromeliads, are invaluable for investigating microbial biodiversity and the possible linkages between terrestrial and aquatic elemental cycling in tropical forests.

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